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Evaluation of ligation methods and the synthesis of a specific PNA-encoded peptide library

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Declaration

This thesis has been composed by the author, and describes research carried out by the author under the supervision of Professor Mark Bradley at the University of Edinburgh from November 2009 till June 2014. Where work has been performed either jointly or wholly by others, this is clearly attributed. No part of this thesis has been previously submitted for any other degree or professional qualification. References have been given where appropriate.

Signed: Date:

Lay Abstract

For doctors to be able to tailor appropriate treatment for individual patients (personalised medicine), it is necessary to analyse and profile specific biological processes involved in the patients' illness. This can be achieved by using a diagnostic kit for a specific disease. For example in leukaemia, the use of patients' biological material to detect and profile the type of cancer they have, would direct the most useful treatment. In this study, a collection of chemical compounds was synthesised with the aim of investigating cancer biomarkers (enzymes) from leukaemia patients.

The compound library, containing "bar code" peptides associated with leukaemia, was designed and synthesised. This barcode can be read using a tool called a microarray. In this thesis, methods of linking the peptide and the barcode were investigated, and the best strategy was employed for the synthesis of the library. A library based on this set-up was tested with cells and analysed. This strategy could be used for analysing other cancers and diseases.

Abstract

Dysfunctional or over and under expressed enzymes play a crucial role in a variety of diseases. A tool that can identify dis-regulated enzymes in individual patients would be beneficial and would allow personalised treatment. For this purpose, a 10,000 membered 'spit-and-mix' PNA-encoded peptide library with a cell penetrating peptide was synthesised and interrogated with K562 cell lysate and intact K562 cells.

This allowed the specific enzyme activity pattern for ABL tyrosine kinase from both inside a cell and a lysate to be obtained. Hybridisation of this library with a DNA-microarray resulted in bio-fouling by the cell lysate, thereby preventing analysis of the phosphorylation pattern. To allow extraction and purification of the peptide library from the cell lysates, a His-tag was incorporated into the library, and enabled successful library analysis.

In addition to this 10,000 member library, a focused 100 PNA-encoded peptide library was synthesised. The library included peptide sequences known to be phosphorylated by specific tyrosine kinases deregulated in acute lymphoblastic leukaemia (ALL) with a PNA-tag complementary to a DNA microarray. Different ligation methods to conjugate the peptides to PNA-tags were screened – this included amide coupling, copper catalysed azide–alkyne cycloaddition, strain promoted azide–alkyne cycloaddition and Diels–Alder cycloaddition. The inverse electron demand Diels–Alder cycloaddition between a tetrazine and norbornene was chosen as the preferred ligation method, and the reaction conditions optimised. To purify the library from cell lysate, a His-tag was again coupled to each member using the strain promoted azide–alkyne cycloaddition.

To test the tetrazine ligation, fluorescence *in situ* hybridisation (FISH) was used in cells, whereby a fluorophore was ligated onto a tetrazine–conjugated PNA probe. This was hybridised onto an mRNA in fixed cells. Results indicated that the ligation needed further optimisation.

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Content

DECLARATION ..... **I**

LAY ABSTRACT ..... **II**

ABSTRACT ..... **III**

ACKNOWLEDGMENT ..... **IV**

CONTENT ..... **V**

LIST OF ABBREVIATIONS ..... **VII**

1. INTRODUCTION **2**

1.1. TYROSINE KINASES 2

1.1.1. Tyrosine kinases and Disease *3*

1.1.2. Personalised Medicine *4*

1.2. PEPTIDE NUCLEIC ACID 5

1.2.1. Applications of PNA *6*

1.2.2. Synthesis of PNA *9*

1.3. INTRODUCTION TO DNA MICROARRAYS 11

1.3.1. Microarrays and Encoded PNA–libraries *13*

1.3.2. Microarrays for clinical use *14*

1.4. LIGATION METHODS IN CHEMICAL BIOLOGY 15

1.4.1. Native Chemical Ligation *15*

1.4.2. Click chemistry *17*

1.4.3. Inverse Electron Demand Diels–Alder Reactions using Tetrazines *19*

1.4.4. Other ligations *21*

1.5. AIMS 23

2. CELL LYSATE ANALYSIS WITH A PNA ENCODED 10,000 MEMBERED SPLIT-AND-MIX LIBRARY **26**

3. EVALUATION OF LIGATION METHODS **41**

3.1. PEPTIDE COUPLING 41

3.2. COPPER(I)-CATALYSED AZIDE–ALKYNE CYCLOADDITION (CUAAC) 42

3.3. NON-COPPER CLICK LIGATION 45

3.4. TETRAZINE 47

3.4.1. 3,6-Bis(3,5-dimethyl-1H-pyrazol-1-yl)-s-tetrazine *47*

3.4.2. Synthesis and test ligation of trans-cyclooctane *50*

3.4.3. Mono-Acid tetrazine *51*

3.4.4. Stability of norbornene during TFA cleavage 58

3.5. CONCLUSION 61

4. EVALUATION OF A PNA *IN SITU* HYBRIDISATION AND FLUOROPHORE LIGATION STRATEGY 63

4.1. INTRODUCTION 63

4.2. RESULTS AND DISCUSSION 69

4.2.1. PNA synthesis 70

4.2.2. In-cell hybridisation and ligation 72

4.3. CONCLUSION 75

5. PNA–PEPTIDE LIBRARY 77

5.1. SYNTHESIS OF PNA-TAGS 77

5.2. SYNTHESIS OF PEPTIDE FRAGMENTS 81

5.3. LIGATION OF PEPTIDES TO PNA-TAGS 90

6. CONCLUSION 97

7. EXPERIMENTAL 101

7.1. GENERAL SECTION 101

7.2. GENERAL EXPERIMENTAL PROCEDURES 103

7.3. CHAPTER 3 106

7.4. CHAPTER 4 124

7.5. CHAPTER 5 127

REFERENCES 148

APPENDIX 163

List of Abbreviations

µW	Microwave
28S rRNA	28S Ribosomal ribonucleic acid
Abl	Abelson
ACN	Acetonitrile
ALL	Acute lymphoblastic leukaemia
Alloc	Allyloxycarbonyl
AML	Acute myeloid leukaemia
anhyd.	Anhydrous
Ar	Arene
BCR	Breakpoint cluster region
BCR–ABL	Breakpoint cluster region–Abelson
Bhoc	Benzhydryloxycarbonyl
Bts	Benzothiazole-2-sulfonyl
CAPS	Cryopyrin-associated periodic syndromes
cDNA	Complimentary deoxyribonucleic acid
CF	Carboxy fluorescein
CM	ChemMatrix
CML	Chronic myeloid leukaemia
CPP	Cell penetrating peptide
CuAAC	Copper-catalyzed azide–alkyne cycloaddition
DAPI	4',6-Diamidino-2-phenylindole
Dde	1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl
DMAP	4-Dimethylaminopyridine

EDC(•HCl)	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (hydrochloride)
EDTA	Ethylenediaminetetraacetic acid
EEDQ	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
ELSD	Evaporative light scattering detector
FAM	Fluorescein
FGFR	Fibroblast growth factor receptors
FISH	Fluorescent *in situ* hybridisation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
HBTU	*N,N,N′,N′*-Tetramethyl-O-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate
HCTU	1-[Bis(dimethylamino)methylen]-5-chlorobenzotriazolium 3-oxide hexafluorophosphate
Her-2/neu	Human epidermal growth factor receptor 2/neuro
HFIP	Hexafluoroisopropanol
HMPA	Hexamethylphosphoramide
JAK	Janus kinase
LAH	Lithium aluminium hydride
LDA	Lithium diisopropylamide
*m*CPBA	*meta*-Chloroperoxybenzoic acid
MLST	Multilocus sequence typing
Mmt	Monomethoxytrityl
M-PER	Mammalian protein extraction reagent
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant staphylococcus aureus

Nor-Flu	Norbornene fluorescein
NTA	Nitrilotriacetic acid
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PEGA	Poly[acryloyl-bis(aminopropyl)polyethylene glycol]
PMA	Phosphomolybdic acid
Rho	Rhodamine
smFISH	Small molecule fluorescent *in-situ* hybridisation
spAAC	Strain promoted azide–alkyne cycloaddition
TAMRA	5(6)-Carboxytetramethylrhodamine
TBAF	Tetrabutylammonium fluoride
TBTA	Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TFE	Trifluoro ethanol
TIPS	Triisopropylsilyl
TIS	Triisopropylsilane
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
Trt	Triphenylmethyl
Tyr2	Tyrosine kinase 2
TZ	Tetrazine
TZ-OH	4-(6(Pyrimidine-2-yl)-1,2,4,5-tetrazin-3-yl) benzoic acid
TZ-OSu	4-(6(Pyrimidine-2-yl)-1,2,4,5-tetrazin-3-yl) benzoic succinamide
VEGFR	Vascular endothelial growth factor

Chapter I

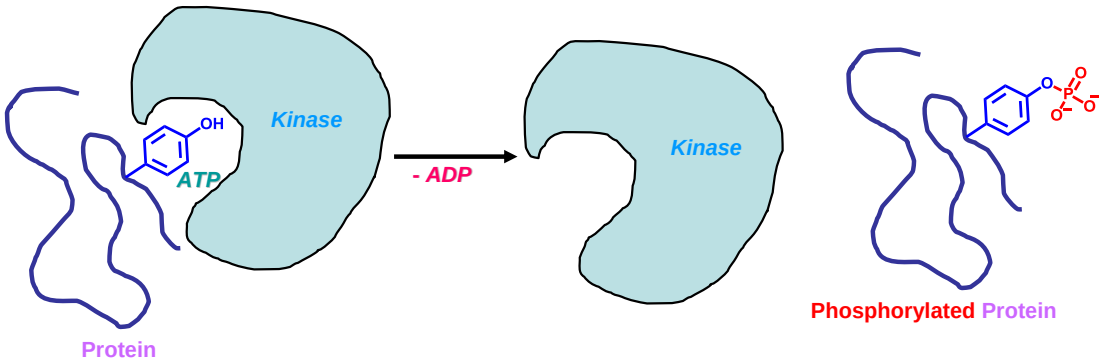
Introduction

1. Introduction

1.1. Tyrosine kinases

Kinases are a family of enzymes that phosphorylate specific substrates. There are three main families the tyrosine, the serine/threonine, and dual kinases (targeting both tyrosine and serine/threonine), but other amino acids such as histidine and arginine are also targeted by kinases. Tyrosine kinases are important mediators of various signalling processes, leading to cell proliferation, differentiation, migration, metabolism and programmed cell death.[1] In healthy cells tyrosine kinases are tightly regulated, with dysfunction due to mutation or overexpression, often leading to malignancy.

There are more than 500 kinases encoded in the human genome, of which 90 are tyrosine kinases.[2,3] These enzymes transfer a *γ*-phosphate group from adenosine triphosphate (ATP) to the target tyrosine, thereby phosphorylating the protein (**Scheme 1.1**). Of the 90 tyrosine kinases, 58 are proteins with a predicted transmembrane domain, which can be grouped into 20 subfamilies based on their kinase domain sequences (kinase domain is the catalytic side of a protein).[4] The remaining 32 kinases are non-receptor or non-membrane spanning tyrosine kinases and fall into 10 subfamilies based on their kinase domain sequences.[3]



Scheme 1.1: Representation of a protein phosphorylation by a tyrosine kinase.

1.1.1. Tyrosine kinases and Disease

Protein tyrosine kinases have been shown to play a key role in many disease states, such as different cancers,[5–7] type 1 diabetes,[8] and rheumatoid arthritis.[9] The dysfunction of kinases results in over or under expression of activity. Three tyrosine kinase families are described below, which illustrate the diversity of malfunctioning enzymes.

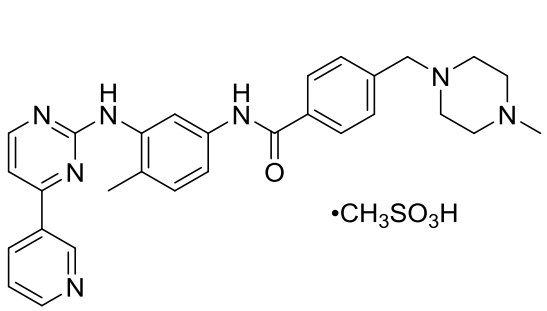
The fibroblast growth factor receptors (FGFRs). There are four members in this family, FGFR 1–4, which are master regulators of a broad spectrum of cellular and developmental processes, migration, proliferation, and apoptosis. Mutations in these enzymes can lead to severe illnesses like cancer[5] or Pfeiffer syndrome.[10]

The vascular endothelial growth factor receptors (VEGFRs). VEGFR 1–3 belong to this family, which regulates blood and lymphatic vessel development and homeostasis. Their dysfunction has shown to play a role in many diseases including diabetic retinopathy, psoriasis, and arthritis. VEGFRs also play a role in cancer where the growth of tumour blood vessels is promoted.[11]

The Janus kinase (JAK) family. JAKs are one of the ten recognised families of non-receptor tyrosine kinases. Its members, JAK 1–3 and tyrosine kinase 2 (Tyk2), are normally localised in the endosome and the plasma membrane due to their association with cytokine receptors.[12] The JAK family plays a critical role in blood formation and the immune response. Mutation of members in this family are related to myeloproliferative disorders, acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), and other leukaemias. These disorders are all related to an overexpression of those enzymes, in contrast, loss of function of some members leads to immunodeficiency.[6]

As kinases play a role in disease, especially in some cancers, treatments targeting kinase activity have been developed to control or even prevent the specific disease from developing further.[7,13–15] A small molecule that targets specific tyrosine kinases in cancer is Gleevec **1.1**, which targets the breakpoint cluster region–Abelson protein (BCR–ABL) that is present in 95% of chronic myeloid leukaemias (CML)

and up to 30% of acute lymphoblastic leukaemia patients (ALL).[7] When added to cells, Gleevec resulted in a 92–98% decrease in the formation of cells that express BCR–ABL with no effect on healthy cells.[16]



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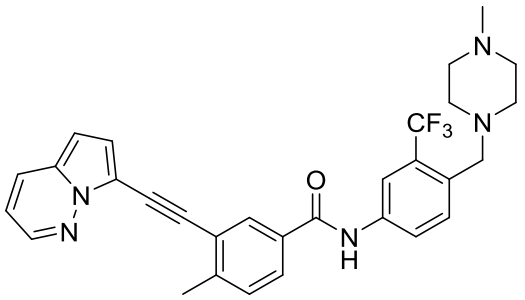


1.2

Another drug in development, is Ponatinib (**1.2**) that targets FGFR protein tyrosine kinases.[13] This inhibitor is currently being explored in clinical trials, which **1.2** also showed some inhibition of BCR–ABL kinase in CML patients. In mice this drug showed a reduction in tumour growth, and importantly showed similar potency as previously observed in the BCR–ABL models.[13] There are many other kinase inhibitors that can be targeted therapeutically and these have been extensively reviewed in the literature.[16,17]

1.1.2. Personalised Medicine

Schleidgen has defined personalised medicine as:

> *"Personalised medicine seeks to improve stratification and timing of healthcare by utilising biological information and biomarkers on the level of molecular disease pathways, genetics, proteomics as well as metabolomics."*[19]

In general, personalised medicine is the improved treatment, for individual patients based on their genetic and family history. The aim is to focus treatment, so that it is known that the intended treatment will work for specific enzymatic mutations.[20] Personalised medicine is becoming an important strategy in patient care, and is employed in the treatment of CML. In 95% of CML,[7] a fusion between chromosome 9 and 22 results in the Philadelphia chromosome, which expresses the fusion oncoprotein BCR–ABL. This enzyme is a tyrosine kinase and can be treated with a tyrosine kinase inhibitor (like Gleevec).[21] This concept has been applied for many different diseases and is continuously growing. Some examples where personalised medicine is used in clinics to date are in cystic fibrosis,[22] various cancers,[21,23] and inflammatory bowel disease,[21] amongst others.

1.2. Peptide Nucleic Acid

Peptide nucleic acids (PNA) are synthetic mimics of deoxyribonucleic acid (DNA) (or ribonucleic acid (RNA)), first described in 1991 by Nielsen.[24] Instead of the phosphate deoxyribose backbone of DNA, PNA is based on a peptide backbone where the nucleobases (guanine, cytosine, thymine and adenine) are attached *via* a methylene carbonyl spacer. Because of its neutral character, PNA hybridises quickly and efficiently with DNA. PNA forms very stable duplexes with complementary single-stranded DNA, RNA and PNA, through Watson–Crick base pairings (**Figure 1.1**).[25] Those complexes can be formed in a parallel (*C*-terminal of PNA facing the 3'-end of DNA) or an anti-parallel (*N*-terminal of PNA facing the 3'-end of DNA) orientation. The anti-parallel orientation is more favourable, showing a higher melting temperature (T_m) compared to the parallel complex.[26,27]

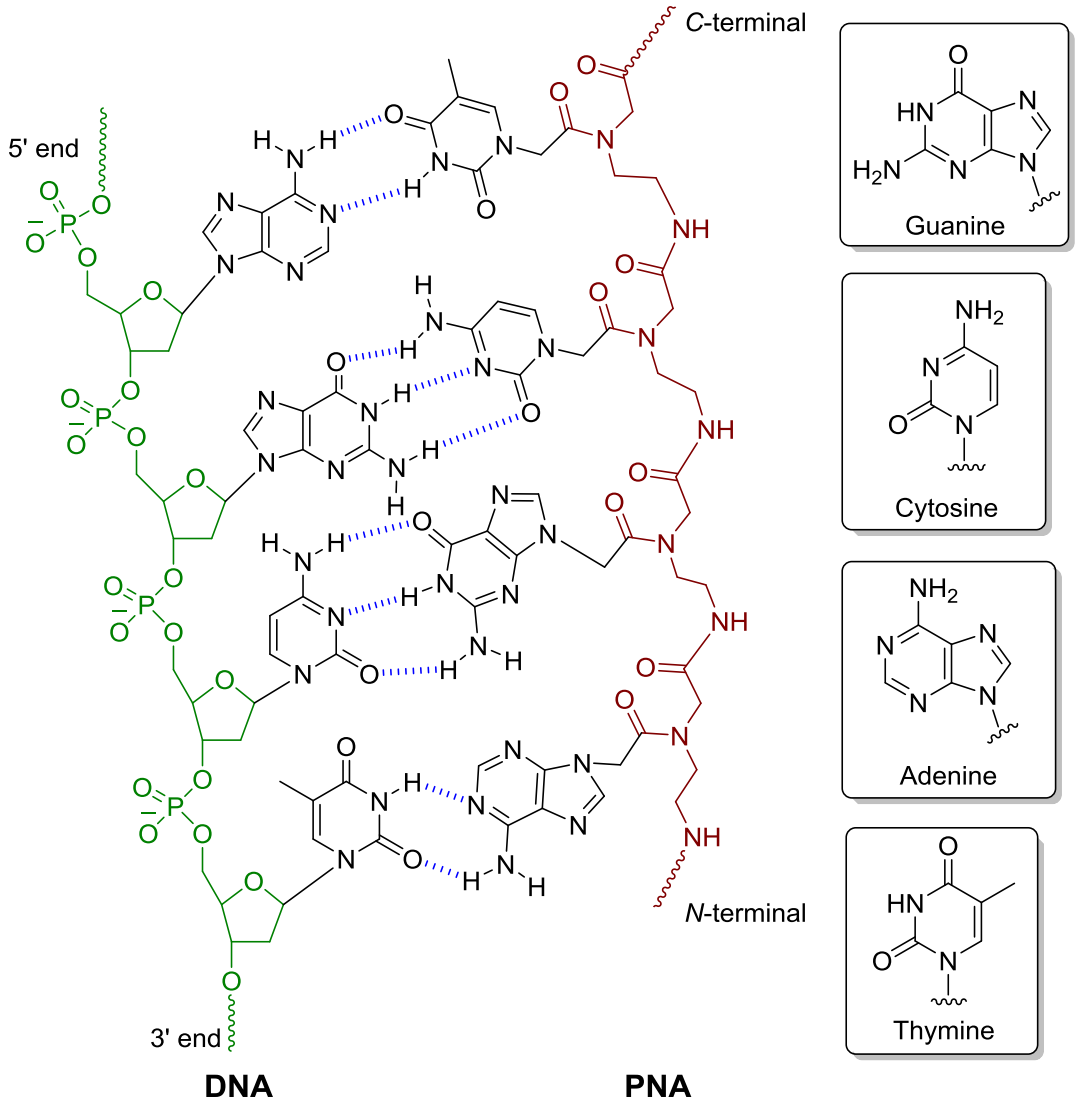


Figure 1.1: Watson-Crick base paring of DNA (left, green) base with the complimentary PNA (right, red) base through intermolecular hydrogen bonding (blue dashed lines).[28,29]

1.2.1. Applications of PNA

PNAs have been used to inhibit the expression of specific proteins by hybridising either to the DNA-target sequence (antigene inhibition) or to a messenger RNA (mRNA) (antisense) (**Figure 1.2**). PNA antigene inhibitors hybridise to a specific DNA sequence in the nucleus forming a PNA–DNA triplex, preventing the transcription of the gene,[27,30] and subsequent protein synthesis. Antisense PNA

inhibitors target mRNA in the cytoplasm forming an unnatural PNA–RNA duplex that cannot be read by ribosomes, hindering the production of a specific protein.[27,31,32]

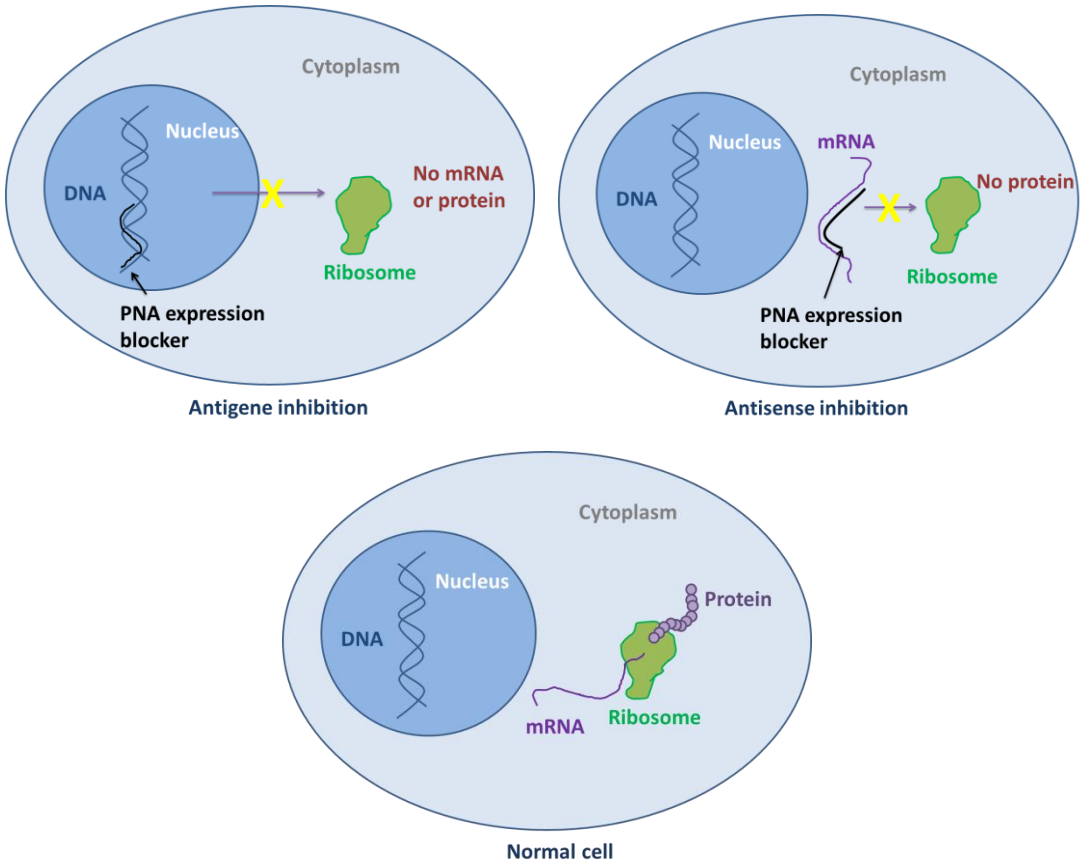


Figure 1.2: Antisense and antigene inhibition by a PNA inhibitor.

Another application of PNA is as biosensors that can detect specific DNA sequences,[30,33] and identify single point mutations.[34,35] The detection of the target DNA can be achieved electrochemically, visually, or by mass spectrometry.[36] Luo *et al.*[33] investigated the detection of DNA using a PNA probe that was not immobilised on the surface of the electrochemical detector. For this assay, a complimentary PNA sequence was synthesised and functionalised with a ferrocene (Fc) moiety and another PNA probe was prepared with methylene blue (MB). Those indicators showed different peak currents and could be used in tandem. When no DNA was

present, a signal was observed, but in the presence of a complimentary DNA sequence, the PNA–DNA duplex no longer bound to the detector surface due to charge interactions (**Figure 1.3**).

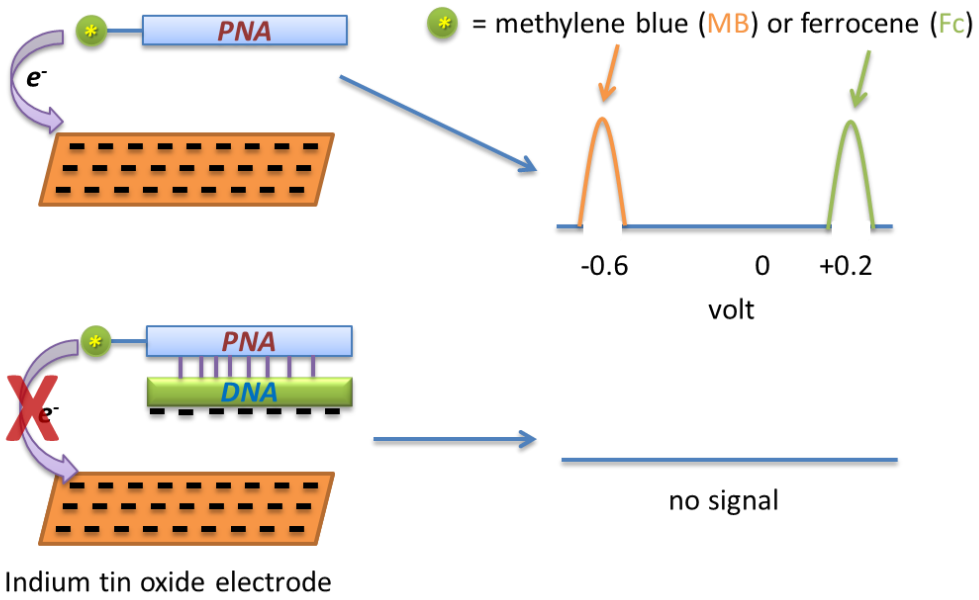


Figure 1.3: Ilustration on how a PNA probe was used by Luo to detect electrochemiclly the presence of a DNA sequence.[33]

A mass spectrometric method was developed by Bowler *et al.*[34,35] to analyse DNA point mutations. In this method, a PNA probe with a blank position opposite the nucleobases of interest was synthesised. This probe was then hybridised with the DNA target, to form a duplex with a blank position in the PNA sequence. To identify what nucleobases were present in the DNA, the four nucleobases were added as aldehydes, and only the complimentary nucleobase was incorporated into the missing position *via* imine formation. After reducing the imine to the amine, the PNA was analysed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI–TOF MS) to identify which nucleobase was incorporated

(**Figure 1.4**). A further development of this method, is the use of fluorescent-labelled nucleobase aldehyde monomers to detect single point mutations.[37]

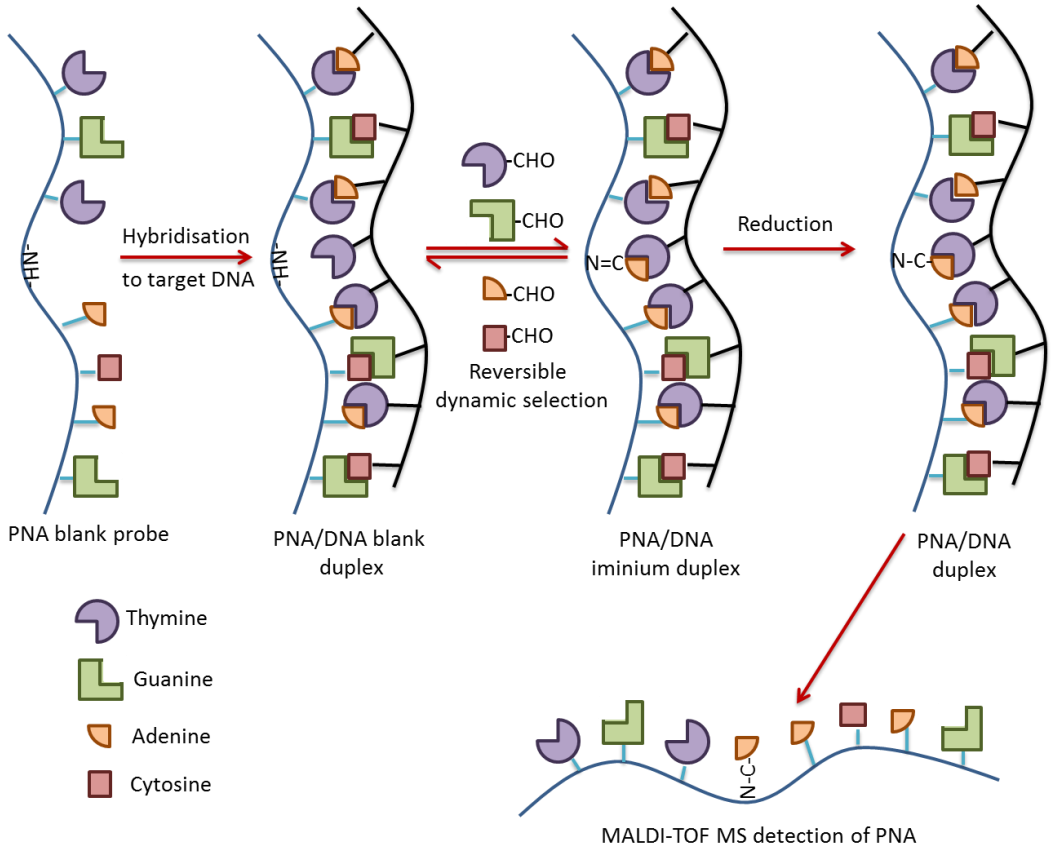


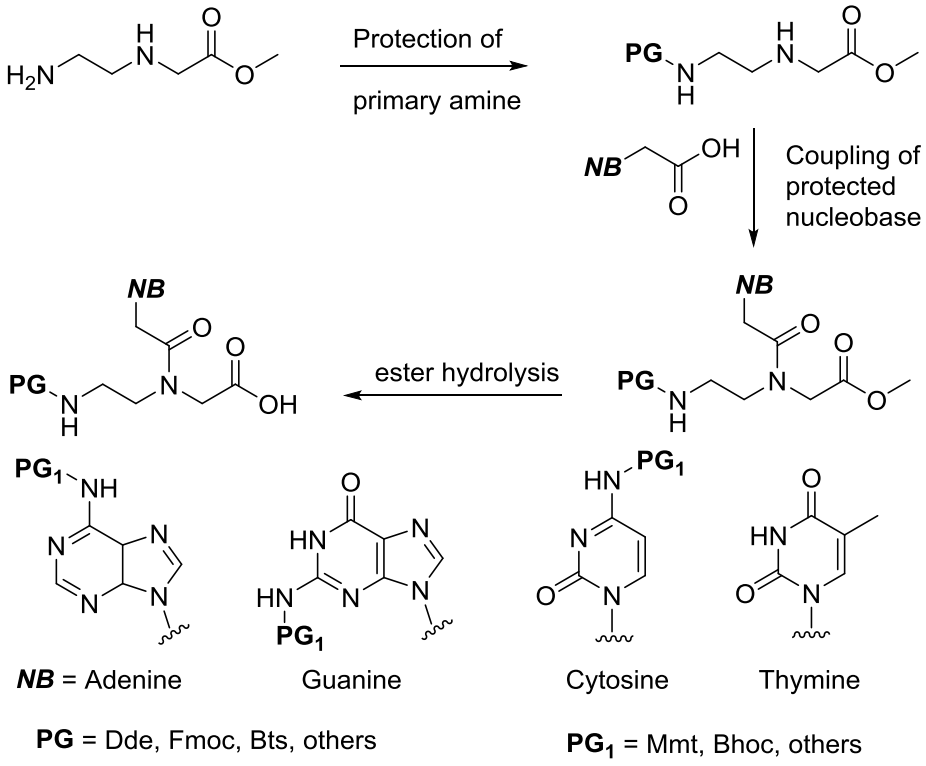
Figure 1.4: Dynamic chemistry applied to the analysis of single point mutations.

Other examples of PNA are as molecular beacons,[27] and fluorescent *in situ* hybridisation (FISH) probes (introduced in depth in **Chapter 3**).

1.2.2. Synthesis of PNA

Standard solid phase peptide synthesis (SPPS), using an Fmoc approach, can be employed to synthesise PNA oligomers.[26,38] In the SPPS approach, the PNA monomers are synthesised with appropriate protecting groups (**Scheme 1.2**) including fluorenylmethyloxycarbonyl (Fmoc), *tert*-butyloxycarbonyl (Boc),

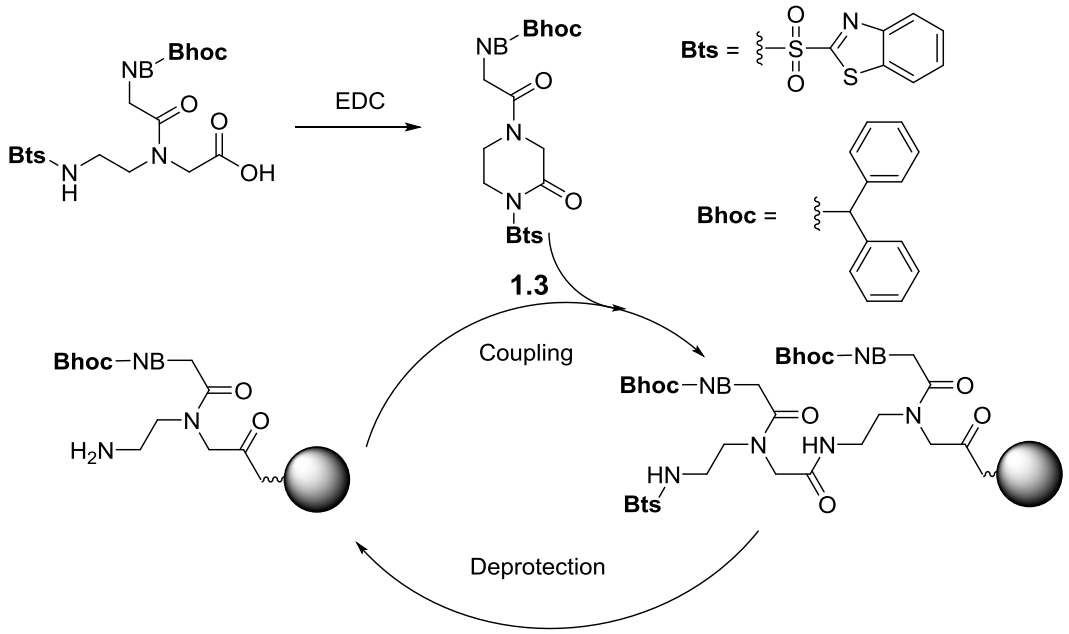
monomethoxytrityl (Mmt), 2-(4-biphenyl)isopropoxycarbonyl (Bpoc) and *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) (Dde) (Dde has been used by the Bradley group extensively in parallel with Fmoc-peptide to synthesise PNA-encoded peptide libraries).[29,39–41] Fmoc/benzhydryloxycarbonyl (Bhoc) protected PNA monomers are commercially available.



Scheme 1.2: Generic synthesis of PNA monomers.

SPPS is typically employed for the synthesis of PNA. The amino acid monomer is first activated to form an active ester, which is then coupled to a free amine on a solid support. To reduce the potential of side reactions and to improve reactivity, Lee[38] reported a cyclic PNA monomer (**1.3**) that is self-activated and efficient in PNA synthesis. In this case, the protecting group on the backbone of the PNA monomer is benzothiazole-2-sulfonyl (Bts). The Bts group being deprotected under mild conditions using 4-methoxythiophenol and *N*,*N*-diisopropylethylamine

(DIPEA). An advantage of using this type of monomer is that no additional compounds are needed for the coupling reaction (**Scheme 1.3**).



Scheme 1.3: Cyclic PNA monomer (**1.3**) synthesis and SPPS described by Lee.[38]

1.3. Introduction to DNA microarrays

Microarrays are small analytical devices that can be used to analyse a large number of analytes in a rapid manner. The word microarray comes from the Greek word *'mikro'*, meaning small, and the French word *'arayer'*, meaning ordered. This technology was first reported by Fodor and co-workers[42] in the early 1990s, (although Southern[43] also has a claim) when a 1024 membered peptide array was synthesised for the first time. The peptides were synthesised on the surface of a slide using light sensitive protecting groups, and screened using a fluorescently labelled antibody.[42] The surfaces used now extend to glass, silicon or nylon, containing hundreds to millions of different compounds (DNA,[44] peptide,[45] polymers,[46] small molecules,[47] tissue,[48] alongside others[49]) in an ordered arrangement on the surface. Microarrays have many different applications. In this section examples are described

to show their scope and breadth, but this is by no mean an extensive review of microarrays.

A peptide microarray was used by Li[50] to detect the protein kinase A (PKA) activity in cell lysates. For this, 10 peptide sequences specific for PKA were first immobilised on the surface of a microarray, followed by the incubation of the array with cell lysate. The detection of the phosphorylation was achieved by using an anti-phosphoserine-biotin antibody, which in turn was detected by a streptavidin gold nanoparticle. Final imaging was by resonance light scattering analysis. The result of this showed that the peptides had different phosphorylation efficiencies, and the best peptide was then used in a different experiment to quantify PKA concentration in different cell lines. The quantification of enzyme in the different cell lines was comparable with the results from the commercial enzyme-linked immunosorbent assay (ELISA) kit.

Hansen[51] synthesised a polymer microarray with 291 different polymers and a positive control collagen to interrogate platelet rich plasma. The aim was to find polymers that would activate the platelets. 12 'hit' polymers were found that activated platelets (confirmed by fluorescent and scanning electron microscopy). One polymer had, after a 30 min exposure, a grater coverage of platelets bound to the spot surface compared to collagen and formed lager aggregates.[51]

The Neri group[52] synthesised a 30,000 membered small molecule DNA encoded library and screened it against human interleukin-2. To synthesise this library, 100 different small molecules were first encoded with a unique DNA sequence. This was followed by mixing and splitting (into 300 pools) before 300 small molecules were reacted to each of the 100 molecules and a second unique DNA code added. The library was then treated with the protein of interest immobilised onto a magnetic bead. After washing away non-bound molecules and eluting the most potential binders, the DNA code was amplified by polymerase chain reaction (PCR) before the 'hits' were analysed using a microarray. The results were validated by resynthesising the most abundant small molecules.[52]

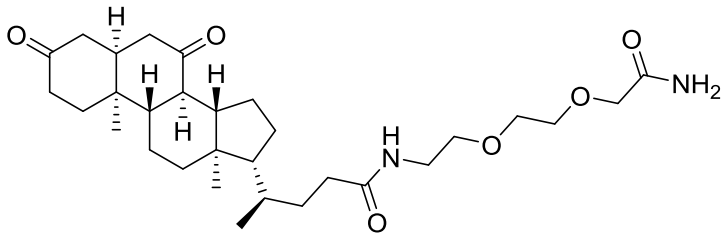
1.3.1. Microarrays and Encoded PNA–libraries

There are benefits to carrying out biological screening of libraries in solution compared to surfaces. This is because enzymes/proteins may interact with the surface, while accessibility may be reduced. Due to this, some groups have synthesised libraries (peptides and small molecules), which are encoded with a PNA-tag, with the entire library decoded by hybridising the library onto a microarray.

For example, Svensen[53] synthesised a PNA-encoded peptide library using a split-and-mix method, with six different amino acids (proline (Pro), lysine (Lys), glutamic acid (Glu), leucine (Leu), tyrosine (Tyr) and lysine like peptoid (Llp)). These were chosen to cover all the different classes of natural amino acids, in addition, the Llp was included. This study was set out to investigate the cell permeability potential of the library. The library was incubated with different cell lines and after trypsin treatment (to remove any peptides and members of the library still on the surface of the cell) the cell lines were lysed. The PNA-encoded library members from inside the cell were collected and purified, and a microarray was used to identify which tetramer peptide showed the ability to enter cells. Surprisingly, the best peptide for all the cells was the negatively charged peptide Glu-Llp-Glu-Glu. This was verified and showed to possess the same or better uptake abilities compared to the positive control. In addition, it was possible to obtain homing peptides that were readily taken up by one specific cell line, but not others.[53] This study shows the power of how all members of a library can be interrogated to obtain huge amount of information in a single experiment.

Daguer[54] used a PNA-encoded small molecule library to find a new binding molecule for streptavidin. In this study, the 62,500 membered library was hybridised onto a DNA-microarray, followed by incubation with labelled streptavidin. After scanning of the microarray, one feature was highly fluorescent. This turned out to be a derivative of dehydrocholic acid (**1.4**), that was highly selective for streptavidin

(K_D = 87 nM).[54] This example shows how a small molecule library encoded with PNA can be used to identify ligands that bind strongly to a specific protein.



1.4

Pouchain[40] synthesised a 10,000 membered PNA-encoded peptide library to interrogate the Abelson tyrosine kinases (Abl). The library was treated with ATP and the kinase and after hybridisation onto a microarray, an anti-phosphotyrosine antibody was added followed by a secondary antibody labelled with a Cyanine 3 dye (Cy3). Scanning the microarray allowed the profiling of the tyrosine kinase activety.[40] With this method it was possible to quantify the phosphorylation for each substrate. 155 peptides out of the 10,000 were strongly phosphorylated (in agreement with known Abl specificity) while new target proteins were identified by comparing the peptide sequences of the 'hits' with a database that contains all the amino acid sequences of human proteins.

1.3.2. Microarrays for clinical use

Microarrays have been used in clinical environments,[55] and some examples are given below.

Balow[56] used a DNA microarray for the profiling of the gene expression of patients with cryopyrin-associated periodic syndrome (CAPS), and compared the expression profile before and after treatment with interleukin-1 inhibitor (anakinra). This study was carried out with 22 CAPS patient and 14 healthy controls to obtain a profile of gene expression for CAPS. 90 genes were identified that can be used to classify CAPS. Using this set of genes, 17 samples from an independent dataset

were correctly identified. Interestingly 15 out of 16 CAPS samples were also correctly identified from patients who had been treated with anakinra despite these patients being in clinical remission.[56]

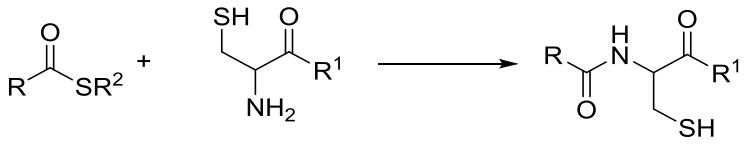
Monecke[57] used a microarray to analyse and identify different methicillin-resistant *staphylococcus aurous* (MRSA) strains encoding the β-pore forming Panton-valentine leukocidin.[57]

1.4. Ligation methods in chemical biology

Chemical ligation is a technique that allows the synthesis of long peptides by joining together smaller fragments. In this project, chemical ligation will be used for attaching individual PNA-tags to peptides. There are several different strategies,[58–61] of which the most popular are discussed below.

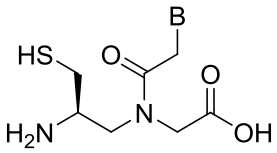
1.4.1. Native Chemical Ligation

Native chemical ligation is the reaction between a *C*-terminal thioester, and an *N*-terminal cysteine residue, to form an amide bond between two peptides (**Scheme 1.4**). Cysteine is a relatively uncommon amino acid (~2%) in nature,[62] therefore introduces a limitation in the synthesis of long peptides, where several smaller peptides[63–65] chains are combined together, although it can be removed by treatment with Raney Nickel.

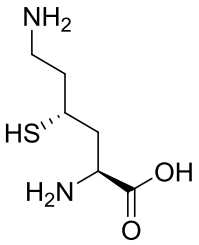


Scheme 1.4: Native chemical ligation reaction between a thioester and cysteine to form an amide bond.

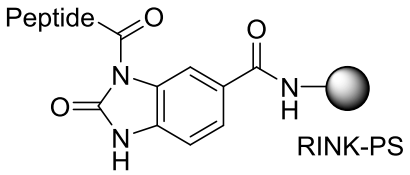
Seitz[59] used this method to couple small PNA chains together, for this a cysteine PNA-monomer analogue was synthesised (**1.5**). A templated native chemical ligation was used, where a DNA sequence acted as the template binding both PNA sequences and bringing the two ends into close proximity.[66–68] On closer investigation, it was determined that using DNA as a template, native chemical PNA ligation was as fast and selective as an enzymatic ligation.[66,68]



1.5



1.6



1.7

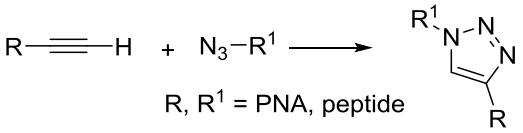
Yang[63] synthesised a lysine derivative in which a thiol group was introduced in the γ-carbon of lysine (**1.6**). Using this monomer it was possible to perform a dual native chemical ligation, with different protecting groups on the two amine functionalities. Using **1.6**, Yang showed that native chemical ligation could be carried out with lysine instead of cysteine, which is more abundant in proteins.

For native chemical ligation, it is necessary to have a thioester peptide, which is challenging when Fmoc-chemistry is used for the peptide synthesis. Because of this, Blanco-Canosa[65] prepared an aromatic *N*-acylurea moiety linker at the *C*-terminus (**1.7**), which could easily be transformed into a thioester by thiolysis. When cleaved, the acylurea moiety on the peptide is stable under acidic conditions, but in neutral aqueous buffers it undergoes rapid thiolysis yielding the thioester on the *C*-terminus of the peptide needed for the ligation. This thioester can also be produced

in situ during the ligation step, allowing the native chemical ligation to proceed relatively efficiently.[65] To test this new linker, a 29 amino acid long rabies-derived peptide was synthesised in two fragments, followed by their ligation. The linker **1.7** shows a smart alternative, which is more efficient than methods used to make thioesters of peptides, as it can be included in the Fmoc-solid phase synthesis.

1.4.2. Click chemistry

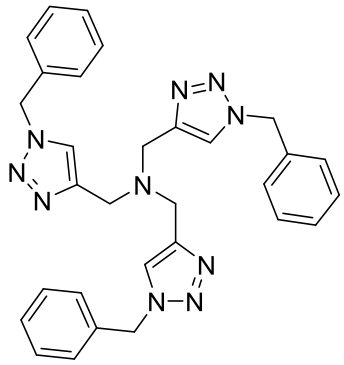
Copper(I)-catalysed azide–alkyne cycloaddition (CuAAC), first described by Meldal in 2002,[60] is the reaction of an azide with an alkyne in the presence of a copper(I) catalyst to form a 1,2,3-triazole. This reaction has since been used in bioorthogonal conjugation to label DNA,[69] material science to synthesise polymers,[70] medical chemistry for the synthesis of biological active compounds,[71] and many more examples. In PNA–peptide chemistry, this reaction can be used to connect PNA and peptide analogues that have previously been functionalised with alkyne and azide moieties (**Scheme 1.5**).



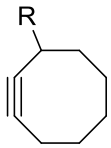
Scheme 1.5: Click chemistry reaction

Two methods have been widely used, amongst others, by Bouillon[72] and Landi[73] who used copper sulphate with sodium ascorbate in a water/alcohol solution for their reactions, while Jolck[74] and Tornoe[60] used CuI and a base in different solvents. Both of these methods have been used on solid support, where the alkyne or azide–peptide is covalently bound to the resin,[60] or in solution phase.[75]

It has been reported that the reaction can be problematic when a copper catalyst is used in the presence of DNA and PNA. Peng and co-workers[76] have shown that in the absence of an additive, DNA is degraded by the conditions used in the copper-click reaction. Fokin *et al*.[77] have shown that the tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand stabilises the Cu(I) oxidation state in water, and used this for the bioconjugation to a virus where a azide/alkyne was introduced into the membrane, which was then stained by adding a alkyne/azide dye. TBTA was also shown to increase the reaction rate of the copper-catalysed click reaction.[78]



1.8



1.9

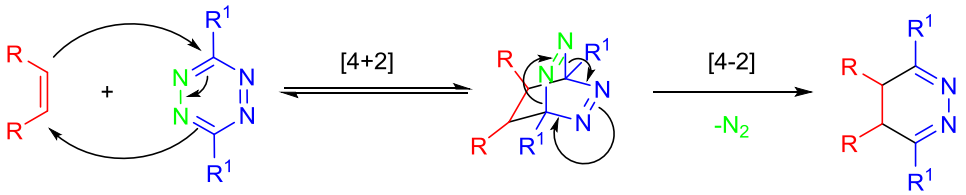
In the past decade, several attempts have been made to use copper or ruthenium free click chemistry. Bertozzi and co-workers reported that strained cyclic alkynes (**1.9**) undergo a [3+2] cycloaddition with an azide without the use of a catalyst.[79] To test the reactivity, the alkyne was reacted with different azides (2-azido-ethanol, benzyl azide, and others), which all showed the only product observed were the two regioisomeric triazoles in approximately equal amounts. Biomolecules have been functionalised with an azide group, followed by the ligation using a biotinylated cyclic alkyne with Western blots used to analyse the reaction to confirm that the reaction was suitable for biological labelling.[79]

Further investigation into strained cyclo-octynes showed that the electron deficient alkyne bond increased the rate of the click reaction. This was achieved by

introduction of two fluorine substituents onto the α-carbon of the alkyne (**1.9**, R = 2 F),[80] and was used by Baskin[80] to label cells. In this work, the Staudinger ligation, a non-fluorinated cyclic alkyne and the fluorinated alkyne were all tested. This showed that the fluorinated compound labelled live cells 20 times faster than the others. This indicates that electron deficient cyclic alkynes react faster compared to just cyclic alkynes.

1.4.3. Inverse Electron Demand Diels–Alder Reactions using Tetrazines

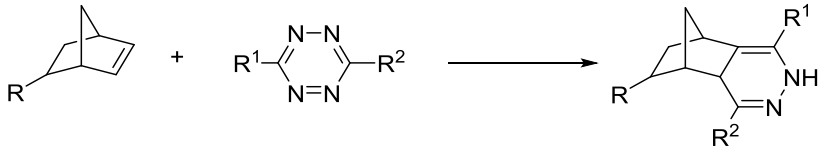
The inverse-electron demand Diels–Alder reaction between a tetrazine and an alkene gives a [4+2] cycloaddition, followed by an irreversible [4-2] ring opening reaction with the release of diatomic nitrogen as the only side product (**Scheme 1.6**).[58,81,82] As it is an inverse-electron demanding Diels–Alder reaction, the diene, in this case the tetrazine, reacts more efficiently when electron withdrawing groups are attached to the tetrazine, and the dienophile is more electron rich, or more strained (*e.g. cis- vs trans*-cyclooctene).[83]



Scheme 1.6: Inverse-electron demand Diels–Alder reaction between a tetrazine and an alkene.

The reaction between a tetrazine and norbornene was used by Schoch[61] to covalently label DNA after oligomer synthesis with fluorescent labels (**Scheme 1.6**). For this DNA oligomers were synthesised that included a norbornene group. This was used to test the effectiveness of the ligation depending on the position on the oligomer. It was shown that the tetrazine ligation was very selective, with yields of 65–80% after only 1 h reaction, and 94–96% conversion after 17 h. There seemed to be no preference of where on the oligo the ligation was carried out, and in contrast to

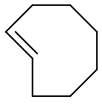
N-hydroxysuccinimide ester coupling and the copper click reaction, this Diels–Alder conjugation showed higher efficiency of labelling with lower reaction concentrations.



Scheme 1.7: Inverse-electron demand Diels–Alder reaction using tetrazine.

Antibodies functionalised with norbornene, and subsequently treated with a near infrared (NIR)-fluorescent dye functionalised with a tetrazine, showed significant ligation of the tetrazine to the antibody after a 30 min incubation period. For the labelling of the A549 (adenocarcinomic human alveolar basal epithelial) cells,[84,85] a *trans*-cyclooctene functionalised antibody was used to detect the epidermal growth factor receptor (EGFR) receptor, which is overexpressed in those cells. After the incubation of the antibody, the tetrazine with a NIR-dye attached, was added to the live cells, and imaged after 10 min. This showed good labelling of the cells. These examples show the potential of tetrazine ligation and labelling of live cells. It was also shown that tetrazines are stable in the buffers used for the ligation and reaction on the cell surface.

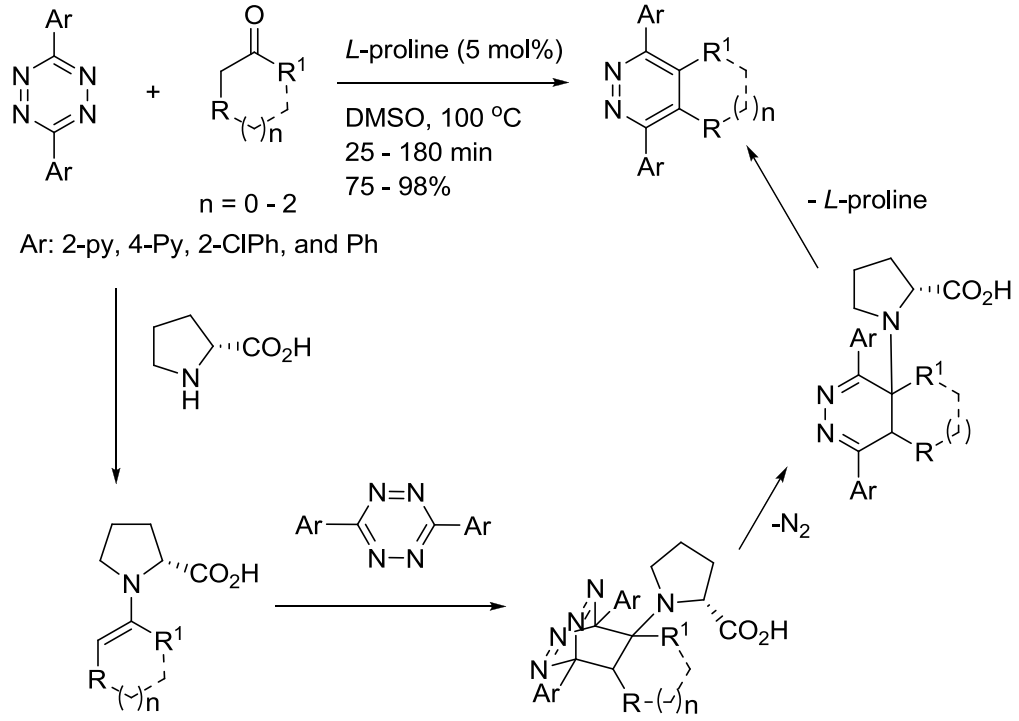
In a study of different dienophiles with tetrazine undertaken by Thalhammer,[83] it has been determined that the fastest Diels–Alder reaction occurred when a *trans*-cyclooctene (**1.10**) was used. This reacted up to six orders of magnitude faster than the corresponding *cis*-isomer, still three orders of magnitude faster than the cyclooctyne.[83] This shows how to tune the reaction with different dienophiles.



1.10

Xie and co-workers[86] investigated a similar type of reaction based on the inverse-electron demand Diels–Alder reaction. Instead of an alkene, a ketone was used to couple with the tetrazine (**Scheme 1.8**). This reaction needed a proline

catalyst to work, as the proline undergoes a Schiff-base reaction with the ketone, forming a double bond, which is the actual reactive dienophile. Using this method, several small molecules were synthesised, which showed the first examples of a Diels–Alder tetrazine reaction in the presence of a catalyst.



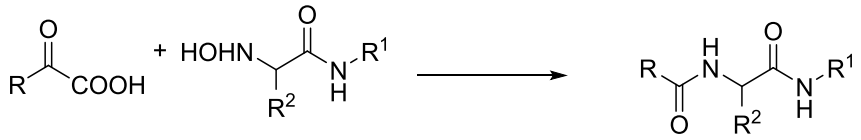
Scheme 1.8: Proline-catalysed direct inverse electron demanding Diels–Alder reactions of ketones with 1,2,4,5-tetrazines.

This was just a small example of the uses of tetrazines, but tetrazines are being increasingly investigated for different ligations from biological labeling[85] to prodrugs[87] and as high energy materials,[88] showing a wide interest in the scientific community.

1.4.4. Other ligations

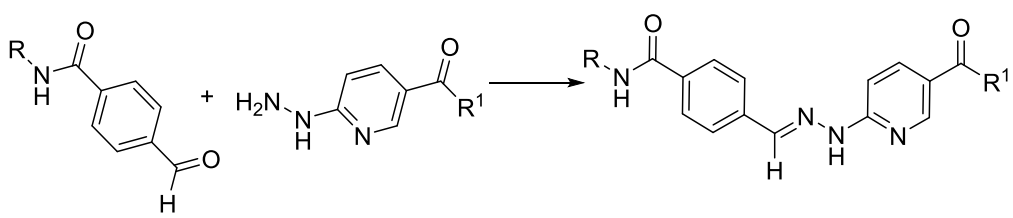
Some other ligation methods used are shown below. One of those ligations is the ketoacid–hydroxylamine ligation, which uses very mild reaction conditions and is very chemoselective. The only by-products are H_2O and CO_2. Bode and co-

workers[89,90] used this to couple two polypeptides together, using no additional reagents. It was carried out in aqueous dimethylformamide (DMF) at temperatures between 37 and 60 °C (**Scheme 1.9**).[91] One of the advantages of this method, over the most commonly used native chemical ligation, lies with the *N*-terminal amino acid used. Any amino acid can be used to couple to the ketoacid of the other peptide, whereas for native chemical ligation it is important to have a sulphur containing amino acid, like cysteine or a modified lysine. The bond formed in this reaction is a normal amide bond, which is useful for the synthesis of medium to large peptides with any sequence desired, and it does not rely on a specific amino acid in the sequence for the ligation to occur.[91]



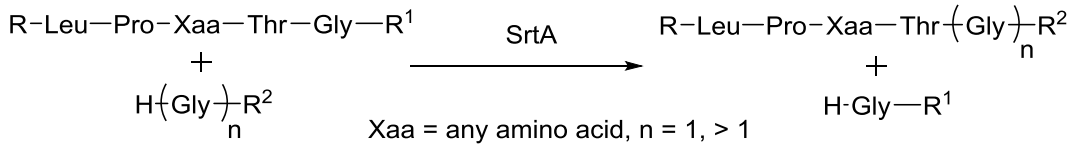
Scheme 1.9: Ketoacid–hydroxylamine ligation

A hydrazone ligation (**Scheme 1.10**) as used by Blanco-Canosa[92] is the reaction between an aldehyde and a hydrazine, which chemoselectively reacts. Aniline can be used as a catalyst. In addition to mild conditions (the reaction carried out at pH 7.0), the peptide side chains do not need to be protected during the ligation. Using this ligation method it was possible to control the ratio of peptides coupled to the surface of a quantum dots as the ligation is selective and high yielding.[92]



Scheme 1.10: Ligation reaction between a 4-formylbenzoyl group and a 2-hydrazinonicotinoyl group.

Enzymatic ligations are another example that can be used for the synthesis of long peptides and proteins. Pritz[91] used the enzyme Sortase A to conjugate a cell penetrating peptide to a PNA sequence. Sortase A is a transpeptidase that cleaves a pentapeptide sequence (**Scheme 1.11**) between the threonine (Thr) and glycine (Gly) residue and transfers it onto the amine of a polyglycine terminated peptide.[91,93]



Scheme 1.11: Reaction mediated by Sortase A (SrtA).

1.5. Aims

The aim of the work in this thesis was to synthesise PNA-encoded peptides. This included a generic split and mix 10,000 membered kinase library, and a 100 membered library to encompass the specific substrates of tyrosine kinases. The libraries were modified by the attachment of a cell-penetrating peptide motif to allow cellular delivery, with the aim of analysing intracellular kinase activity, and a poly-histidine tag. The histidine (His)-tag was designed to allow the isolation of the library from crude cell lysates following interrogation of enzyme activity in cancer samples from patients. The PNA-encoded libraries were designed to be analysed using a customised DNA-microarray to thus determine which enzymes were functional/over-active dysfunctional. The thesis thus reports:

I. Screening and synthesis of a new 10,000 membered PNA-encoded peptide library with enhanced scope for tyrosine kinase substrates compared with a previously used library;[40]

II. Optimisation of library screening methods with cell lysates, and purification of the library using a His-tag;
III. Intercellular kinase activity screening, by addition of a cell penetrating peptide to the 10,000 membered library for internalisation;
IV. Synthesis of 100 PNA tags and 100 peptides using SPPS;
V. Optimisation of the ligation of the PNAs and peptide library,
VI. PNA-based FISH analysis;
VII. Screening of different hybridisation conditions for the DNA-microarray analysis of the library; incubation of the library with purified enzymes to validate the tool using cell lysate from leukaemia cells to interrogate enzymatic activity with and without inhibitors present during incubation.

Chapter II

His-tag split-and-mix library

The work presented in this chapter was carried out in collaboration with Dr Eftychia Koini (University of Edinburgh), Dr Mazen Sleiman (University of Edinburgh), Dr Ruben van Boxtel (University Medical Center Utrecht) and Dr Koen Braat (University Medical Center Utrecht) individual contributions are indicated throughout the chapter.

2. Cell lysate analysis with a PNA encoded 10,000 membered split-and-mix library

This section of the project was based on previously published work by Bradley,[40] where a 10,000 membered tyrosine kinase library was synthesised using different amino acids (acidic, basic, hydrophilic and hydrophobic). This library was screened with several tyrosine kinases to identify their preference for amino acids surrounding a central tyrosine residue (**Figure 2.1**).

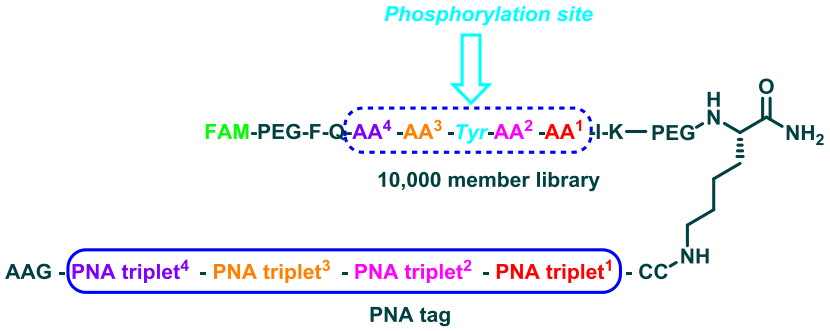


Figure 2.1: Peptide library as published by Bradley.[40]

The library was synthesised using the 'split-and-mix' approach first reported by Furka in 1988.[94,95] This technique allows for the synthesis of large libraries using relatively few coupling steps. As the name suggests synthesising a split-and-mix library means that the "synthesis resin" is split into portions before the different amino acids are coupled (and in our case their PNA-codes). Once coupling of the amino acids and PNAs are complete, the *split* resin samples are combined and thoroughly *mixed* before being *split* again into equal portions prior to the next amino acid coupling (**Figure 2.2**).

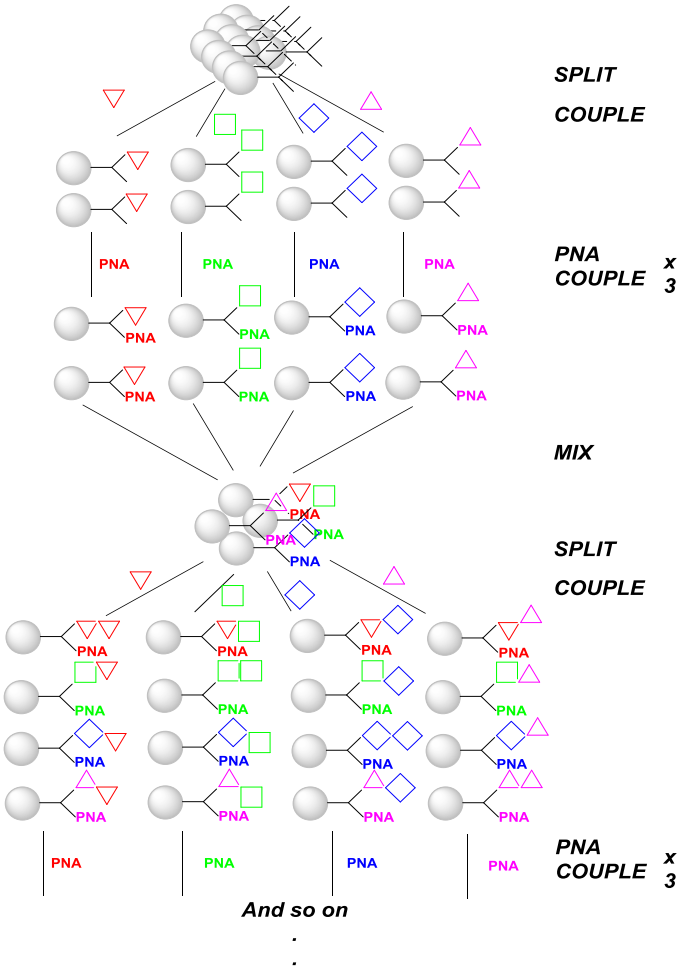


Figure 2.2: Representation of a split-and-mix synthesis method used for a PNA-encoded peptide library. First the resin was split into four equal amounts, followed by the coupling of the first amino acid, and its corresponding triple PNA code. The split resins were mixed together, and split again into four equal batches. The next amino acid was then coupled with its corresponding PNA-codes. The mixing, splitting and coupling steps where repeated until the desired number of couplings was achieved.

With this method a new library containing 10,000 members was synthesised using only 4 split-and-mix cycles, with a total of 40 couplings for the varying amino acids and 120 couplings for their corresponding PNA codes. Data base searching showed that the old library covered about 5% of all the known tyrosine kinase recognition sequences. Thus a new library was designed to cover about 20% of all known tyrosine kinase sequences (**Table 2.1**) and synthesised[A] on polyethylene glycol polyacrylamide copolymer (PEGA) resin (0.2 mmol/g).

[A] Library synthesis on Fmoc-Rink-PEGA resin by the author and Dr Eftychia Koini.

Table 2.1: PhosphoSitePlus® database analysis of amino acids around the phosphorylation site of all known tyrosine kinases. Numbers are shown as a percentage, with the yellow representing the ten most abundant amino acids used for the synthesis of the new library.

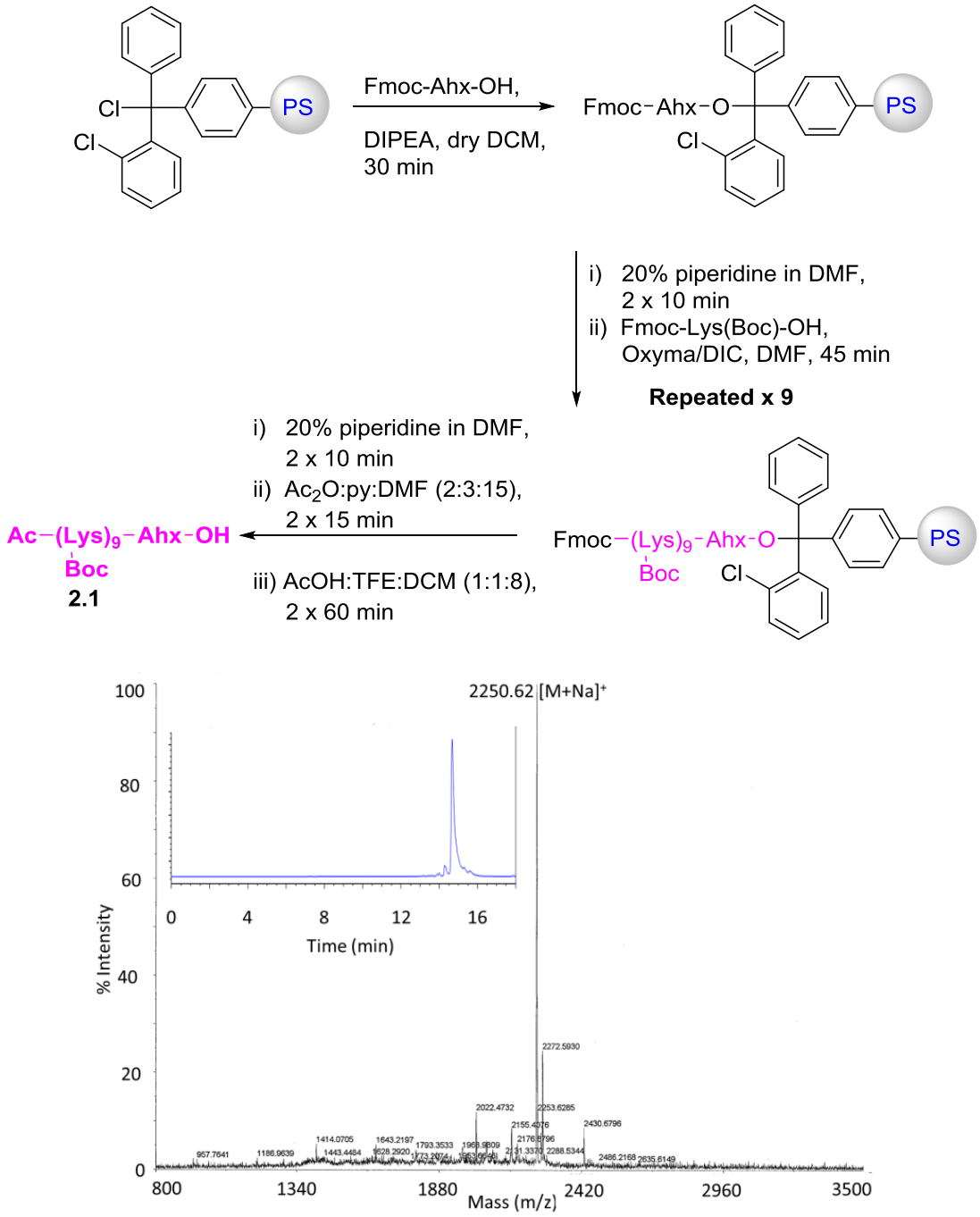
Amino Acid	*Position -2 (%)*	*Position -1 (%)*	*Position 0 (%)*	*Position 1 (%)*	*Position 2 (%)*
Alanine	*6.97*	*6.00*	*0*	*6.47*	*6.65*
Arginine	*5.61*	*4.75*	*0*	*4.94*	*5.40*
Asparagine	*4.75*	*4.63*	*0*	*3.76*	*5.00*
Aspartic Acid	*7.80*	*8.70*	*0*	*6.40*	*6.76*
Cysteine	*1.20*	*1.31*	*0*	*1.50*	*1.84*
Glutamic Acid	*7.51*	*7.48*	*0*	*8.94*	*7.53*
Glutamine	*4.15*	*3.94*	*0*	*5.28*	*4.14*
Glycine	*7.80*	*6.70*	*0*	*6.61*	*6.58*
Histidine	*2.21*	*2.54*	*0*	*2.58*	*2.07*
Isoleucine	*3.48*	*6.20*	*0*	*4.46*	*4.24*
Leucine	*7.16*	*7.73*	*0*	*7.75*	*7.35*
Lysine	*6.56*	*5.62*	*0*	*5.08*	*6.12*
Methionine	*1.86*	*1.58*	*0*	*2.08*	*2.52*
Phenylalanine	*2.35*	*2.62*	*0*	*2.99*	*2.80*
Proline	*7.75*	*5.85*	*0*	*3.48*	*5.65*
Serine	*8.97*	*7.47*	*0*	*10.46*	*8.95*
Threonine	*5.10*	*5.61*	*0*	*5.46*	*5.98*
Tryptophan	*0.49*	*0.62*	*0*	*0.62*	*0.64*
Tyrosine	*3.29*	*3.77*	*100*	*4.38*	*3.58*
Valine	*4.94*	*6.89*	*0*	*6.49*	*5.54*

= Amino acids used for the improved library.

For this library, an analysis of all known tyrosine kinase substrates was undertaken and the 10 amino acids with the highest abundance for the two positions on either side of the phosphorylation site were chosen.[B] A cell penetrating peptide (CPP **2.1**, Lys_9) was also added to the library to allow the library to enter cells in order to enable analysis of the enzyme activity inside a cell.

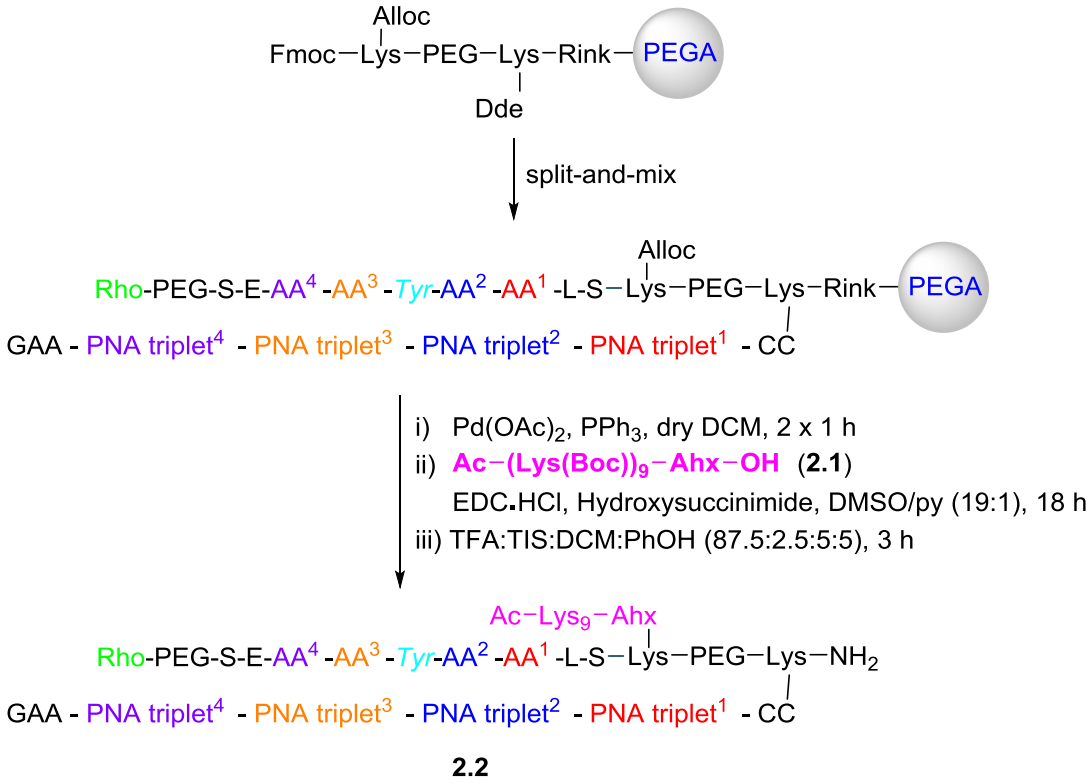
To ensure this to be achieved a CPP was synthesised on a 2-chlorotrityl functionalised polystyrene resin and cleaved as shown in **Scheme 2.1**.

[B] Analysis done by Dr Ruben van Boxtel.



Scheme 2.1: Top: Synthesis of protected CPP **2.1**. Pre-activated 2-chlorotrityl polystyrene resin was functionalised with Fmoc-Ahx-OH (Fmoc-aminohexanoic acid) in the presence of DIPEA. Followed by Fmoc-deprotection using piperidine and coupling of Fmoc-Lys(Boc)-OH using Oxyma/DIC. After repeating the coupling nine times, the CPP was capped with acetic anhydride and cleaved from the linker with AcOH:TFE:DCM. **Bottom:** MALDI-TOF MS spectra and HPLC (insert) of **2.1**.

The protected peptide was then coupled to the PNA encoded library using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrogen chloride (EDC•HCl) and *N*-hydroxysuccinimide as the last step before cleavage from the Rink-linker (**Scheme 2.2**).



Scheme 2.2: New PNA-encoded library synthesised (split-and-mix method) on PEGA resin using Fmoc-chemistry and Oxyma/DIC covering 20% of known tyrosine kinase substrates and coupling of the CPP (**4.1**). Amino acids used: A, D, E, G, K, L, N, P, R, S.

The library **2.2** was added to both cell lysate and K562 (human erythromyeloblastoid leukaemia) cells (**Figure 2.3A**) to obtain a specific fingerprint of kinase activity in cells. However, when the library was hybridised onto the microarrays and scanned, the entire surface of the microarray 'lit-up' in the Cy5 signal (used for phosphorylation information) thus interfering with the analysis of this experiment (**Figure 2.3C**). In contrast **Figure 2.3B** shows the hybridisation control, where the library **2.2** was incubated with a rec. Abl kinase, followed by hybridisation onto a microarray. No contamination was visible on the surface of this array, and from the expansion it can be seen that some of the spots are yellow, meaning information of phosphorylation could be extracted. This observation was

unexpected and was thought to be due to proteins from the cell adhering to the microarray surface.

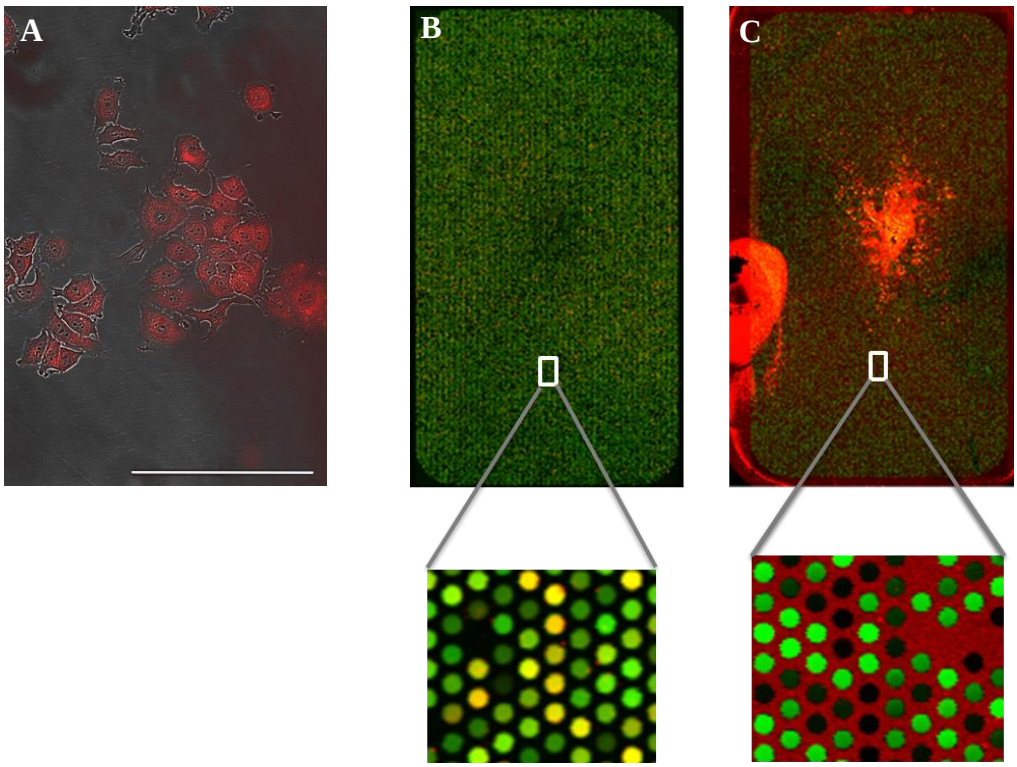


Figure 2.3: A: Showing K562 cells after incubation of library **2.2** (2 µM) in media for 2 h. Merged image of bright field and fluorescent: λ_{ex} = 527-563 nm; λ_{em} = 570-650 nm. Bar = 200 µm**; B:** Image of microarray with library **2.2** treated with rec. Abl kinase hybridised, showing no contamination of the surface between the spots, the yellow spots show phosphorylation information (Cy5 signal)**; C:** Image of a microarray following hybridisation of library **2.2** from cell lysate from K652, showing proteins stuck onto the microarray with the expansion showing the surface between individual spots showing fluorescence. **B and C:** A custom DNA arrays (4 × 44k) from Agilent was used, and hybridisation preformed in GenHyb buffer (genetix). Hybridisation at temperature decreasing from 60 °C to 50 °C in 5 °C steps every 30 min, followed by 2 °C steps every 30 min to 40 °C and finally at 37 °C for 18 h. Green signal of TAMRA, red signal Cy5. [C]

To overcome this problem, the library (with an average weight of 10,000 Da) was partitioned between 30,000 and 3,000 Da filters to remove as much of the cell protein debris as possible. Unfortunately, some of the library was not recovered from the 30,000 Da filter. After extensive washing of the filter, some of the library

[C] Work by Dr Ruben van Boxtel.

was still left on the filter (filter was coloured red). Therefore, results obtained from this library would not give an accurate measure of enzyme activity, as there was no possibility of knowing what was left on the filter. [D]

To reduce the high background signal, various detergents were investigated to try and wash away the protein debris. **Figure 2.4** shows a comparison of the hybridisation signal of the library **2.2** when treated with three different detergents. **Figure 2.4a)** shows the control experiment where the library was hybridised directly, and **b) – d)** show the hybridisation using Tween 20, NP40 and Triton-X as detergents respectively.

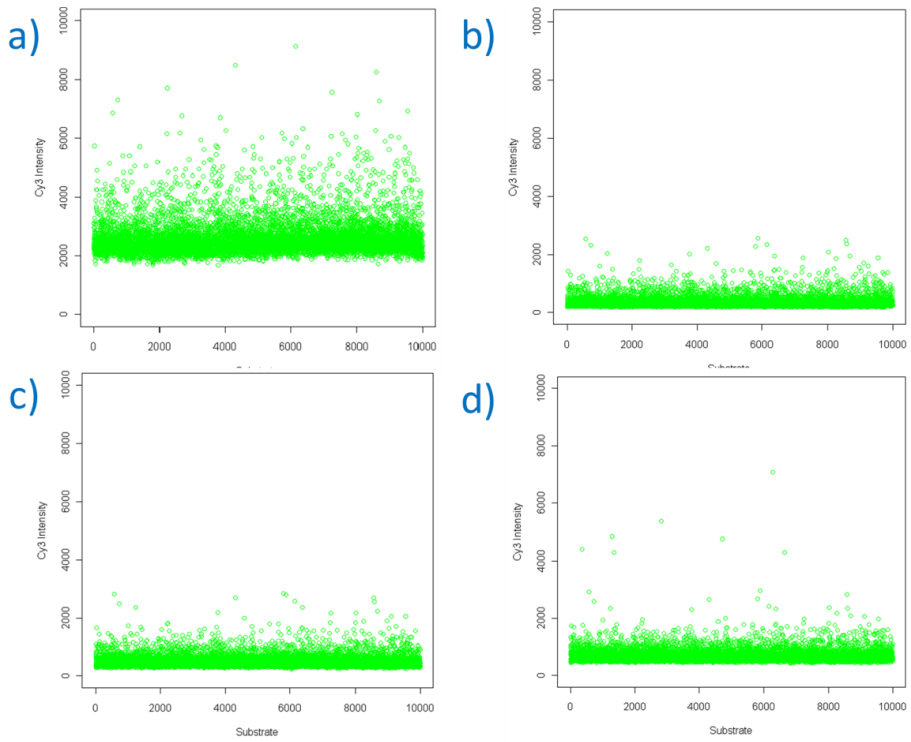


Figure 2.4: Test of the different detergents and their effects on hybridisation. To K562 cells were added Tween 20, NP40 and Triton-X for 10 min, followed by the addition of library **2.2** and incubation for 2 h at 37 °C in the presence of protease/phosphatase inhibitors. After centrifugation, the supernatant was used for the hybridisation. **a)** Library **2.2** hybridised without lysate and detergent; **b)** Tween 20; **c)** NP40; and **d)** Triton-X. The signal produced by TAMRA (which all members of the library possess) was used to determine the efficiency of the hybridisation. [E]

[D] Work by Dr Ruben van Boxtel.
[E] Microarray experiment carried out by the author and Dr Ruben van Boxtel. Data analysed by Dr van Boxtel.

Compared with the positive control, where the majority of the population was at a Cy3 intensity between 2000–4000 units, all the detergents were signals less than 2000. Since the same amount of library was used for all the experiments, it clearly showed that these detergents affected the hybridisation of the PNA-encoded library.

Thus, a different method for collecting or purifying the library from the cell debris was required. For this purpose, a His-tag was chosen and introduced into the library. The His-tag was first investigated by Hochuli[96] in the late 80's and has been hugely used since its discovery for protein purification.[97–99] The imidazole group of histidine binds to Ni(II), which is immobilised on a solid support. Consequently any material without a His-tag can be washed away, before the library is released from the support.

Different cleavage cocktails were tested for the qualitative cleavage of a fully protected Fmoc-His-tag peptide from 2-chlorotrityl linker (see **Table 2.2**). In this study, different concentrations of hexafluoroisopropanol (HFIP) and trifluoroacetic acid (TFA), and a mixture of trifluoroethanol (TFE) and acetic acid, were compared. The evaporative light scattering detector (ELSD) signal from high pressure liquid chromatograph (HPLC) was chosen to evaluate which conditions were best for the cleavage of His-tag so that all the trityl protection groups stayed intact. A fully protected His-tag is important because unprotected side chain amines would introduce side-products and interfere with the reaction when the activated ester His-tag is coupled to the resin bound peptide. Sinapinic acid matrix used for MALDI-TOF MS, is acidic enough, and has been shown, to deprotect the acid sensitive protection groups when spotted onto the MALDI-plate, and because of this the MALDI-TOF MS spectra could show partially unprotected peptides even if none were present originally.[100]

Table 2.2: Screening of cleavage conditions for fully protected Fmoc-His-Tag from 2-chlorotrityl linker.

20% HFIP in $CHCl_3$	*10% HFIP in $CHCl_3$*	*20% HFIP in $CHCl_3$**	*10% HFIP in $CHCl_3$**
79%	*97%*	*79%*	*97%*
0.1% TFA 0.1% TIS in DCM**	***0.1% TFA 0.1% TIS in DCM****	***TFE:AcOH:TIS:DCM (1:1:1:7)***	
81%	*87%*	*97%*	

Percentage of fully protected His-tag product peak area under the ELSD-HPLC chromatogram; HFIP cleavage reaction and TFE each for 2 × 30 min; TFA 3 × 10 min.
* = combined cleavage solutions evaporated with toluene;
** = combined cleavage solutions evaporated with hexane.

From **Figure 2.5**, it can be seen that 20% HFIP (HPLC trace **Right**) reduced the amount of fully protected His-tag (79%) compared to 97% of desired peptide when just 10% of HFIP (HPLC trace **Left**) was used.

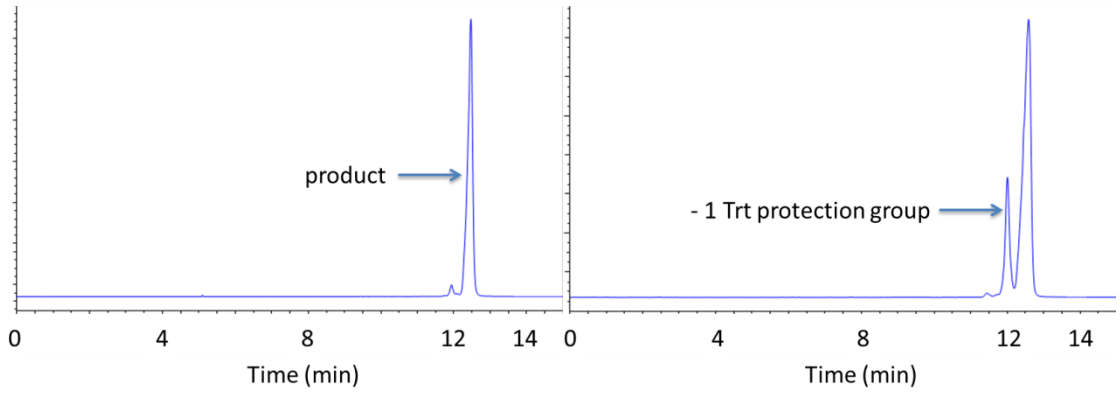
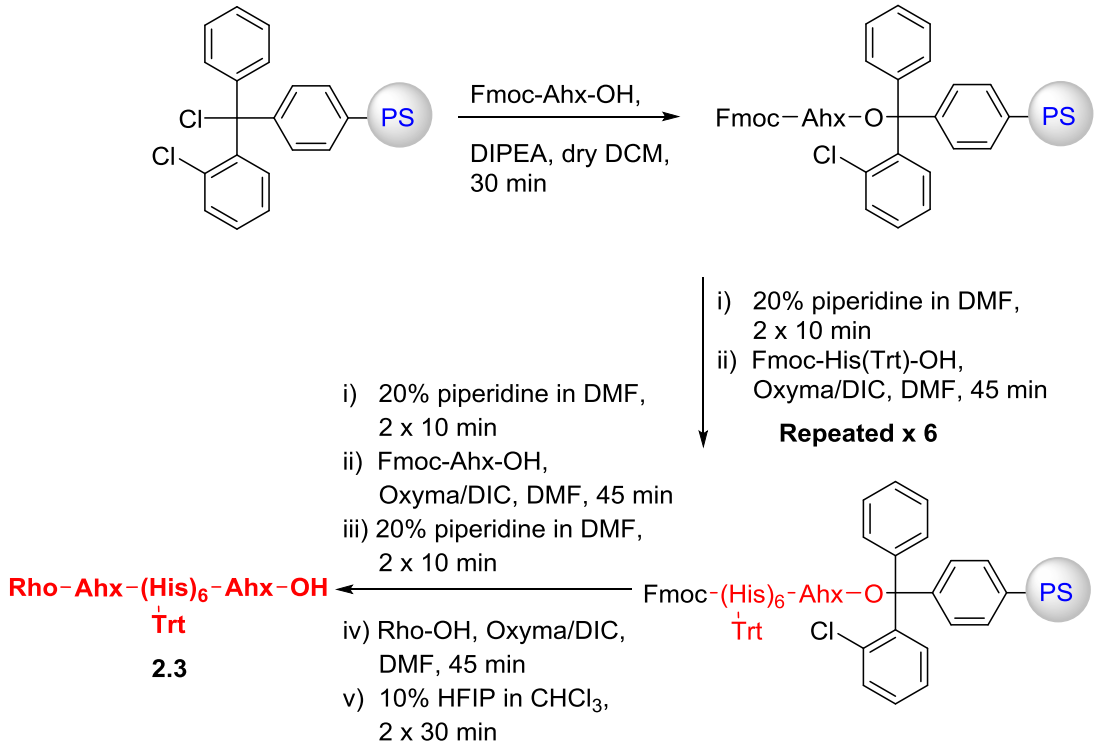


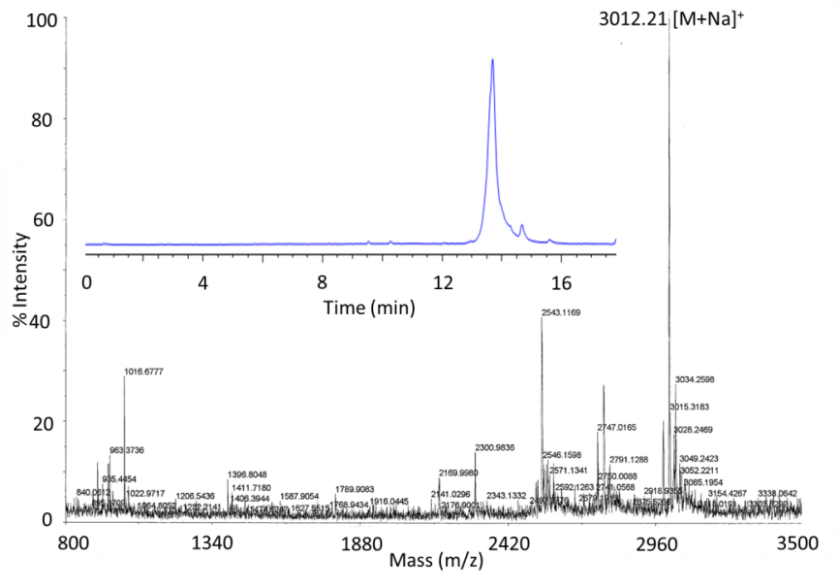
Figure 2.5: ELSD-HPLC trace of test cleavage for protected Fmoc-His-tag showing the fully protected peptide (**Left**) with 10% HFIP and an additional peak for the His-tag with one Trt group being deprotected (**Right**) when 20% HIFP was used.

Using TFA resulted in the cleavage of some of the protecting groups, but never exceeded in more than 80% of successful cleavage/deprotection. The milder conditions of TFE:AcOH showed the same result as 10% HFIP. In addition to the different conditions, alternative work-ups were investigated. For HFIP, the cleavage solutions were either evaporated after addition of $CHCl_3$ or toluene, but both of those did not show any difference in ELSD signal. As for TFA, the solvents used for the work-up were hexane or toluene. A 6% decrease of the mono unprotected side product was detected in the ELSD when hexane was exchanged for toluene. This could be due to the higher boiling point of toluene compared to hexane. As the side chain protection groups of the protected peptide were acid sensitive, a higher concentration of acid should be avoided. In the case when hexane was used as the work-up solvent, hexane evaporated at a faster rate than acetic acid and therefore increased the possibilty of losing some of the protection groups of the peptide. Toluene has a similar boiling point as acetic acid and therefore would evaporate at a similar rate (azeotropes), thereby avoiding the build-up of acetic acid during work-up and deprotection of the peptide. From these test reactions, 10% HFIP in $CHCl_3$ cleavage condition for the protected His-tag with the C-terminal acid functionality intact was chosen.

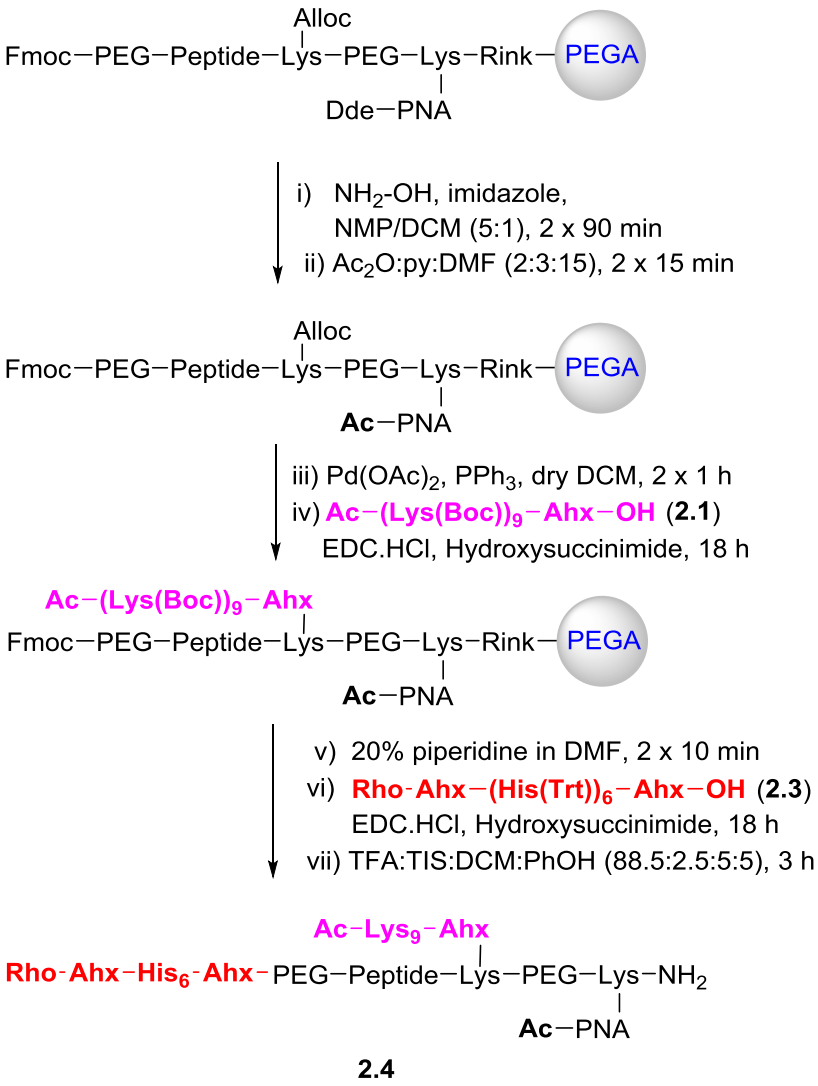
The His-tag with Rhodamine (Rho) attached **2.3**, was synthesised on a 2-chlorotrityl linker attached to a polystyrene resin (**Scheme 2.3**). Compound **2.3** was cleaved from the linker using 10% HFIP in $CHCl_3$. During the synthesis of the new 10,000 library of **2.2** half of the resin was set a side with the Fmoc/Dde/ allyloxycarbonyl (Alloc) protection still intact. To this resin, the CPP **2.1** was added *via* the lysine side chain after de-protecting the Alloc group with $Pd(PPh_3)_4$, while the His-tag was coupled onto the Fmoc deprotected amine using EDC•HCl and *N*-hydroxysuccinamide (**Scheme 2.4**).[F]

[F] Library synthesised by myself, Dr Mazen Sleiman and Dr Eftychia Koini.





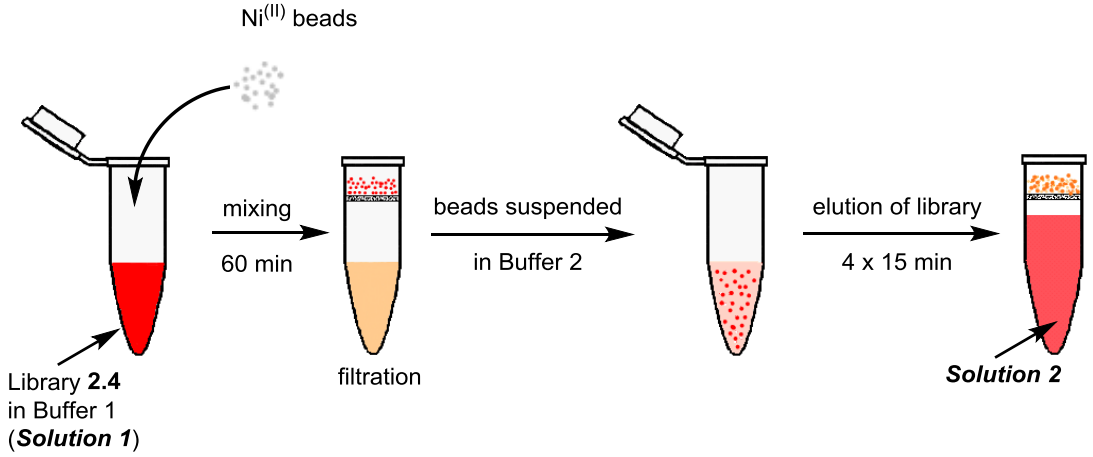
Scheme 2.3: Top: Synthesis of protected Rho-His-tag **2.3**. Pre-activated 2-chlorotrityl polystyrene resin was functionalised with Fmoc-Ahx-OH in the presence of DIPEA. Followed by Fmoc-deprotection using piperidine and coupling of Fmoc-His(Trt)-OH using Oxyma/DIC. After repeating the coupling six times, Fmoc-Ahx-OH was coupled, followed by 5(6)-carboxyl aminoethyl rhodamine coupling. Final cleavage of the protected Rho-His-tag **2.3** from the linker using AcOH:TFE:DCM. **Bottom:** MALDI-TOF MS spectra and HPLC (**Insert**) of **2.3**.



Scheme 2.4: Coupling of His-tag (**2.3**) and CPP (**2.1**) to a 10,000 membered split-and-mix PNA encoded peptide library. The resin was deprotected using H_2N-OH to allow selective cleavage of the Dde-group in the presence of the Fmoc-group, and capped the PNA sequence using Ac_2O. The Alloc group was deprotected using $Pd^{(II)}$ followed by coupling of **2.1**. To couple **2.3**, piperidine was used for the deprotection of Fmoc, followed by addition of His-tag **2.3**. TFA:TIS:DCM:PhOH was used for the cleavage of **2.4** from the Rink-linker and protection group removal.

To test the His-tag purification (**Scheme 2.5**), a small amount of library **2.4** was dissolved in 200 µL of Buffer 1 (20 nM 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 150 nM NaCl, 5% Glycerol, pH 8). The library was incubated for 1 h

with 10 µL of Ni-beads. The beads turned from colourless to red, and were filtered off. The library was eluted by adding 200 µL Buffer 2 (20 nM TRIS, 150 nM NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8) to the Ni-beads, and agitating for 4 × 15 min. The combined filtrates gave 82% recovery of the library.



Scheme 2.5: Library **2.4** test purification using $Ni^{(II)}$ column. **2.4** was dissolved in Buffer 1, $Ni^{(II)}$-beads were added mixed for 60 min. The red beads were filtered, and after washing, suspended in Buffer 2. After 15 min of shaking the beads were filtered and washed with Buffer 2 (× 3), and the filtrates were combined.

Dr Koen Braat mixed the library **2.4** with lysate of human embryonic kidneys cells transformed with large T antigen (HEK 293T, 50 µg), 2× kinase buffer, protease/phosphatase inhibitor and mammalian protein extraction reagent (M-PER), and incubated for 4 h. After incubation the library was purified using $Ni^{(II)}$-(nitrilotriacetic acid) NTA agarose beads. After overnight incubation of the mixture with the beads at 4 °C, the beads were washed, and the library eluted for 90 min at 4 °C. The library was then mixed with the hybridisation buffer (GenHyb), and denatured at 90 °C for 10 min. After this the solutions were added to the microarray and hybridised (**Figure 2.6**).

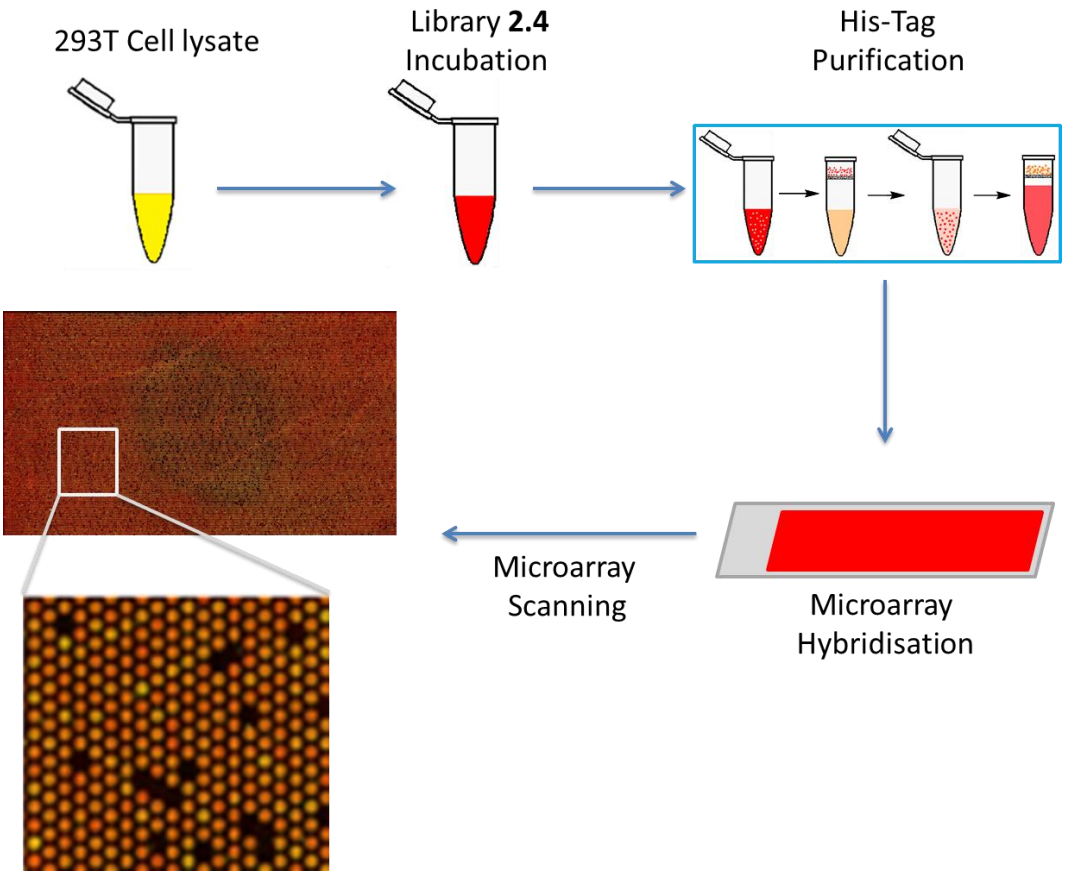


Figure 2.6: Experimental setup showing the incubation, purification and hybridisation onto a custom DNA microarray from Agilent (4 × 44k) of library **2.4** with 293T cell lysate. Image of microarray showing: TAMRA in red and Cy5 in green; with the yellow spot showing members of the library being phosphorylated.[G]

From the microarray image in **Figure 2.6** the cell protein contamination did not appear again, and phosphorylation information could be obtained, showing that the His-purification was successful.

In summary, it has been shown that a His-tag purification of the PNA-peptide library prior to hybridisation on the microarray is suitable for library isolation from a complex cell lysate. It was also possible to deliver the library into cells by the addition of the CPP.

[G] Image and experiment by Dr Koen Braat.

Chapter III

Evaluation of Ligation Methods for PNA–Peptide Conjugation

3. Evaluation of Ligation Methods

To evaluate the best method for ligating a PNA library to a library of peptides several different ligation strategies were investigated. These ligation reactions were:

i. Standard amide coupling.
ii. Copper catalysed azide–alkyne cycloaddition (copper-click, CuAAC).
iii. Copper-free click reactions with an azide and a strained cyclic alkyne (spAAC).
iv. Diels–Alder reaction between a tetrazine (TZ) and an alkene.

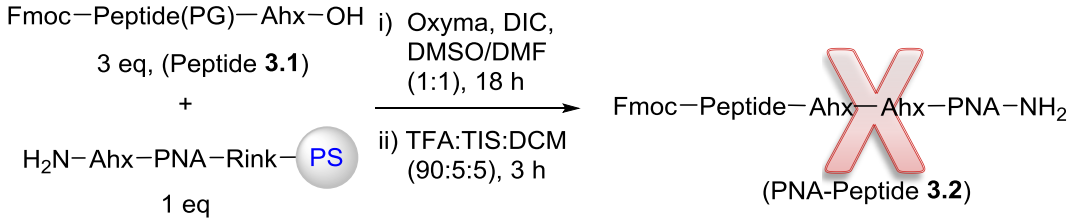
After the evaluation of the different reactions, the most promising was selected and optimised for the synthesis of the PNA–peptide library.

3.1. Peptide Coupling

A fully protected peptide (**Pep035**, **Appendix 2**) was synthesised using a 2-chlorotrityl linker attached to polystyrene resin. The linker is acid labile and when treated with a mixture of TFE:AcOH:DCM (1:1:8), peptides are cleaved from this linker with all the side chain protecting groups intact. In addition, the peptides will be obtained with an acid functionality at the *C*-terminus, allowing coupling to an amine *via* a standard amino acid coupling methods.

For the test reaction, (**Scheme 3.1**) Oxyma and DIC were used to pre-activate the acid group of the protected peptide **3.1** by forming an active ester. After 10 minutes pre-activation, the peptide (3 eq) was reacted with the free amine group of a PNA (**PNA100**, **Appendix 1**) (attached to a resin *via* a Rink linker). After the coupling reaction a Kaiser test was inconclusive, it was not clearly positive or negative. Cleavage and analysis of the PNA–peptide **3.2** by MALDI-TOF MS and HPLC (ELSD detection) indicated that conjugation had not occurred, with only the unmodified PNA recovered. This may have been due to steric hindrance, as the fully protected peptide **3.1** and PNA fragments are very large and bulky (3484 g mol^{-1} and

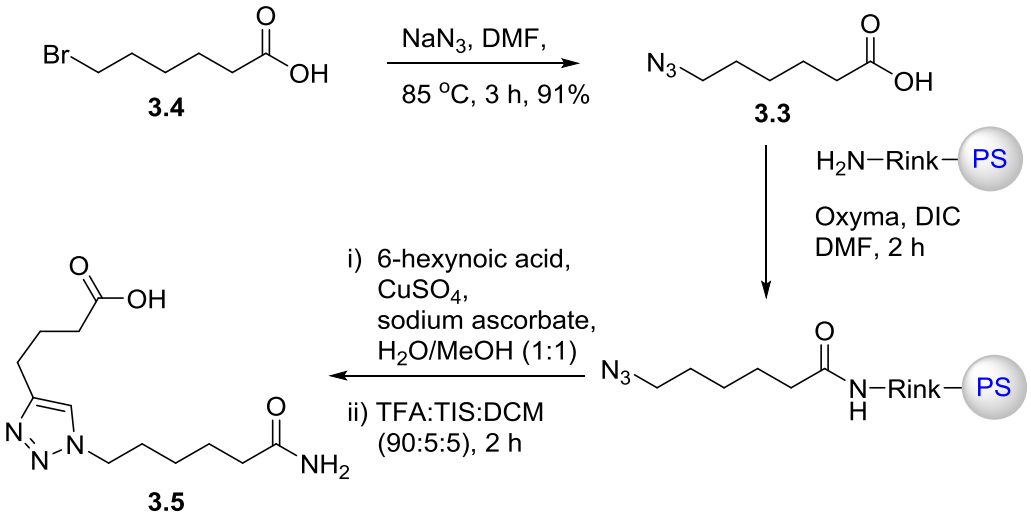
6183 g mol^{-1}, respectively), while the polystyrene resin also had a reasonably high loading of 0.5 mmol/g.



Scheme 3.1: Peptide ligation test using a fully protected peptide acid and a PNA attached to a Rink linker on a polystyrene-resin.

3.2. Copper(I)-Catalysed Azide–Alkyne Cycloaddition (CuAAC)

6-Azidohexanoic acid **3.3** was readily synthesised from commercially available 6-bromohexanoic acid **3.4** and sodium azide in 91% yield, and then coupled directly onto a Rink linker-PS resin. To this resin 5-hexynoic acid was coupled using $CuSO_4$ and sodium ascorbate, and the product cleaved with TFA (**Scheme 3.2**). Material analysed showed the formation of the desired product **3.5** in a 91% crude yield with 9% of the cleaved azide still present (**Figure 3.1**).



Scheme 3.2: Synthesis of 6-azidohexanoic acid and test click reaction on solid support.

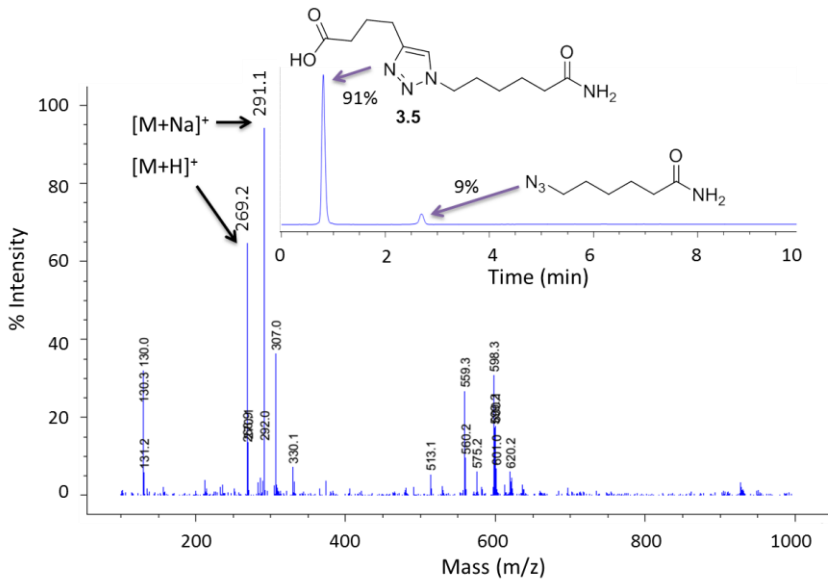
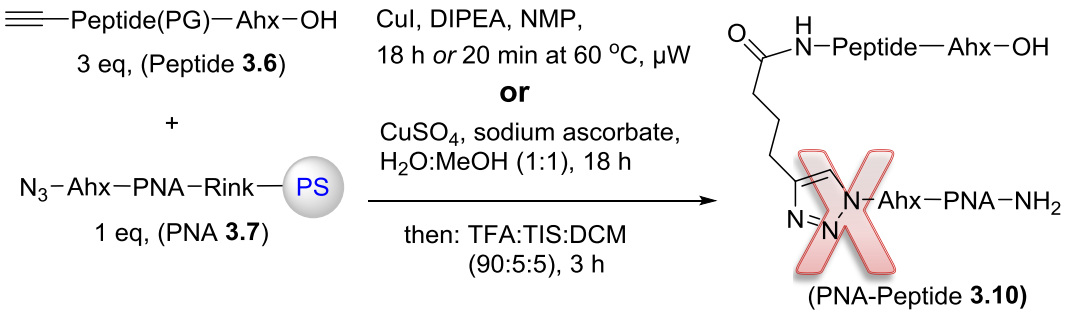


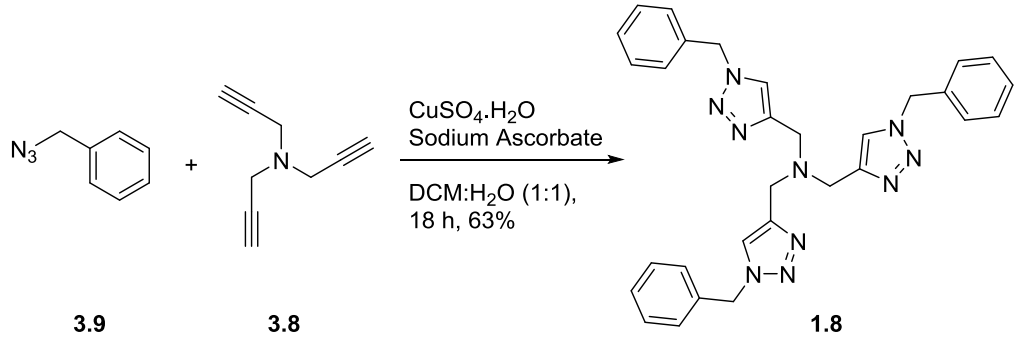
Figure 3.1: LCMS and HPLC with ELS detection spectra of crude product **3.5** from CuAAC ligation test (**Scheme 3.2**).

Following this positive result, an alkyne functionalised peptide **3.6** was prepared and coupled to the azide functionalised beads, again using $CuSO_4$ and sodium ascorbate. Initially, peptide **3.6** was reacted with an azide containing PNA bound to the resin (PNA-resin **3.7**) under several different conditions as shown in **Scheme 3.3**.[71,101] Unfortunately, cleavage from the linker showed only the azide functionalised PNA (**3.7**).



Scheme 3.3: Copper(I)-catalysed azide–alkyne cycloaddition ligation using different standard conditions.

Burrows and Muller[102] reported that copper ions can interact with DNA, and therefore lower or inhibit the copper from catalysing the reaction. To overcome these problems and stabilise the Cu(I) ions, Chan synthesised a ligand that binds strongly to copper(I).[78] This ligand, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, **1.8**), was easily synthesised in one step from tripropargyl amine **3.8** and benzyl azide **3.9**, using $CuSO_4$ as a catalyst in 63% yield (**Scheme 3.4**).

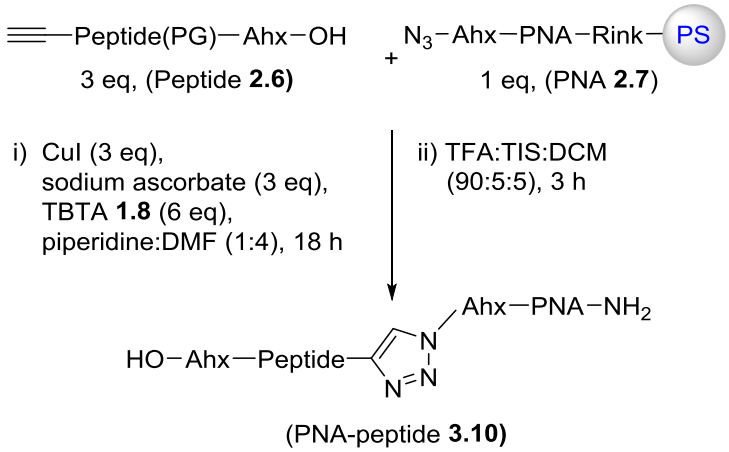


3.9 **3.8** **1.8**

Scheme 3.4: Synthesis of TBTA **1.8.**

Alkyne peptide **3.6** and azide–PNA-resin **2.7** were reacted in the presence of TBTA **1.8** (6 eq), CuI (3 eq) and sodium ascorbate (3 eq). After 24 h, the resin was sequentially washed with DMSO, a solution of TBTA in piperidine:DCM (1:4), DMF, and DCM to remove as much of the copper as possible. Subsequent cleavage from the resin and purification by HPLC resulted in the desired adduct **3.10** in an overall yield of 40% (**Scheme 3.5**). Since copper can interfere with biological reactions,[103] it was crucial to determine the overall copper concentration after the washing and purification steps. The copper concentration in the PNA–peptide **3.10** was determined by inductive coupled plasma-mass spectrometry to be 1.57–1.82 ppb (0.025–0.029 µM). Kennedy[103] has studied the toxicity of different copper complexes in different cells, and showed that the copper toxicity is dependent on the ligand present. The compound which was the most toxic was shown to be so at a concentration of 0.74 µM, which is higher than what was determined from this

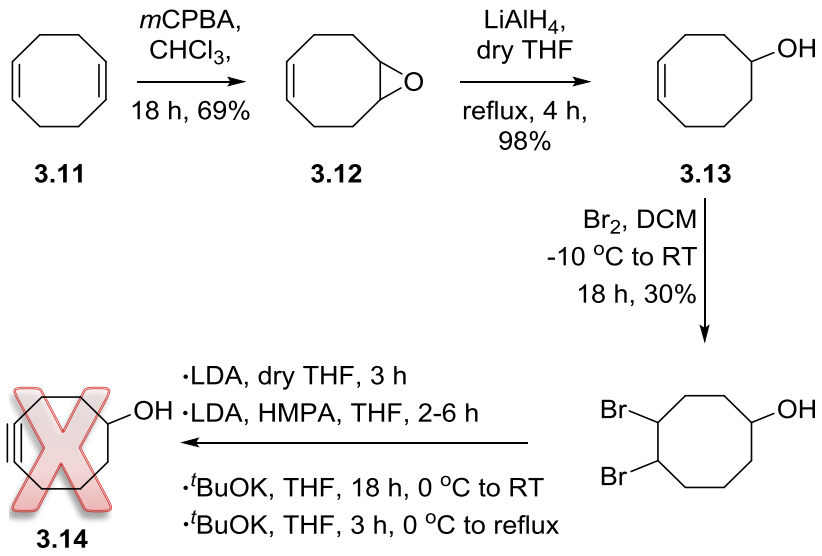
ligation experiment, but as there is some copper present, the CuAAC was not used for ligation as a precaution.



Scheme 3.5: CuAAC ligation using the tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, **1.8**) ligand.

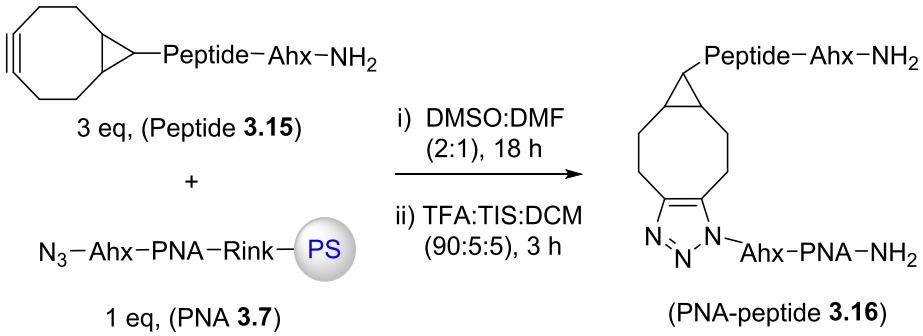
3.3. Non-Copper Click Ligation

Following on from the copper catalysed click reaction, Agard *et al.*[79] developed an azide–alkyne cycloaddition reaction without the need of a catalyst. This required the synthesis of the strained cyclic alkyne **3.14** (4-cyclooctyn-1-ol) (**Scheme 3.6**). 1,5-Cyclooctene **3.11** was converted to the mono epoxide **3.12** with *meta*-chloroperoxybenzoic acid (*m*CPBA). The epoxide was then opened with $LiAlH_4$ to give the *cis*-cyclooctenol **3.13**,[104] followed by di-bromination using Br_2 in DCM.[105] A dual elimination step would generate the triple bond **3.14**. Although several different reactions for the final step were tried (see **Scheme 3.6**), no product was identified, possibly due to product volatility.



Scheme 3.6: Synthesis of 4-cyclooctyn-1-ol **3.14** from 1,5-cyclooctene **3.11**.

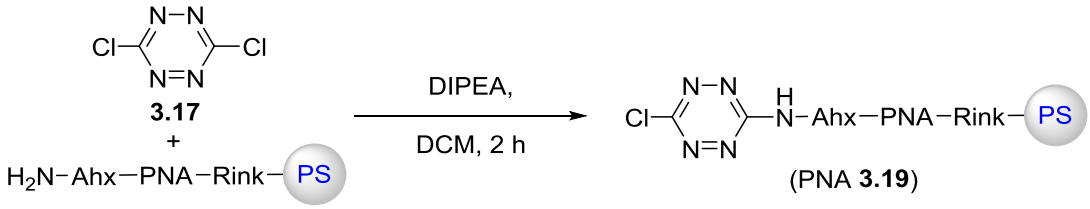
The commercially available bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate was thus used, which was coupled to the free amine of the test peptide **3.15**. After cleavage, peptide **3.15** was coupled to the resin bound azide–PNA **3.7**. The resin was washed to remove the excess peptide, and the peptide–PNA conjugate cleaved to give the desired ligation product **3.16** (**Scheme 3.7**). Analysis of the crude product mixture by MALDI-TOF MS and HPLC, found the product was formed in 28% yield with 59% of the unreacted azide–PNA also present.



Scheme 3.7: Ligation test of copper-free click reaction using the strained cyclooctyne and azide.

3.4. Tetrazine

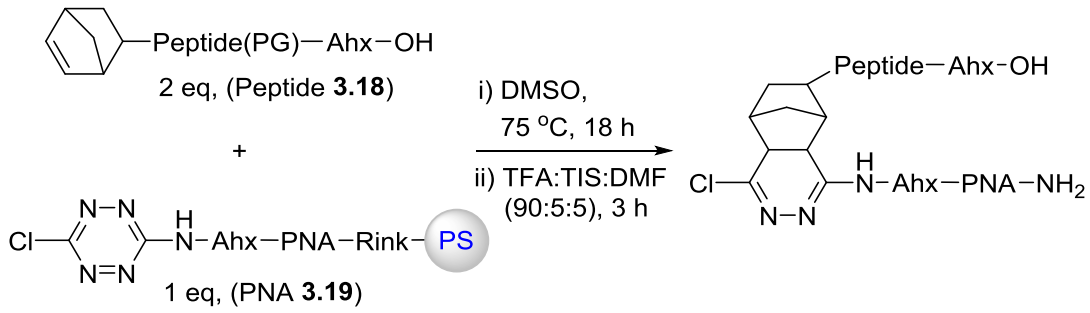
The last ligation strategy tested was based on the inverse electron demand Diels–Alder reaction between a tetrazine (TZ) and alkene. The test TZ used was 3,6-dichloro-*s*-tetrazine **3.17**,[§§] which was prepared in the Bradley group using the synthesis described by Gong (**Scheme 3.10**).[106] This tetrazine was coupled *via* a nucleophilic aromatic substitution of one chlorine to the free amino terminal of the PNA-resin (**Scheme 3.8**), by dissolving the TZ **3.17** in DCM with 2 eq of DIPEA.



Scheme 3.8: Dichloro-TZ **3.17** addition to the amino terminal of the resin bound PNA.

3.4.1. 3,6-Bis(3,5-dimethyl-1H-pyrazol-1-yl)-s-tetrazine

Peptide **3.1** was functionalised with a 5-norbornene-2-carboxylic acid using standard SPPS coupling conditions on a trityl linker. After cleavage, peptide **3.18** was added to the Cl-TZ-PNA **3.19** resin at 75 ˚C (**Scheme 3.9**). Analysis of the cleaved material showed that the reaction had not gone to completion, with starting material (TZ-PNA) present by HPLC analysis, suggesting that the functional groups were not reactive enough.

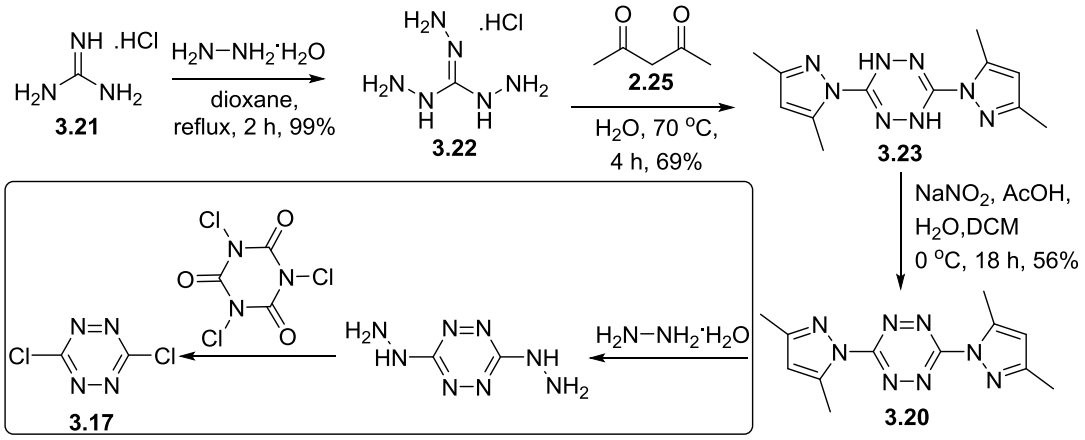


Scheme 3.9: Ligation test of norbornene-peptide **3.19** and Cl-TZ-PNA **3.20** on resin.

[§§] Synthesised by Elizabeth Jameson

Two different ways to improve this reaction were explored: using a more electron poor TZ and introducing an alkene that was more reactive. As the reaction temperature was already at 75 ˚C, greater heating was not further investigated.

3,6-Bis(3,5-dimethyl-1*H*-pyrazol-1-yl)-*s*-tetrazine **3.20** is a precursor to the 3,6-dichloro-*1,2,4,5*-tetrazine **3.17**, and can be coupled to the *N*-terminus of the PNA through nucleophilic aromatic substitution. The synthesis of this tetrazine was easily achieved in high yields by employing commercially available starting materials (**Scheme 3.10**). Guanidine hydrochloride **3.21** reacted with hydrazine in two hours to give triaminoguanidine monohydrochloride **3.22** quantitatively. The adduct formed was used without purification to produce 3,6-bis(3,5-dimethylpyrazol-1-yl)-1,4-dihydro-*1,2,4,5*-tetrazine **3.23** in good yields (69%) by reacting it with 2,4-pentanedione **3.24**. The oxidation of the dihydro-*s*-tetrazine **3.23** to *s*-tetrazine **3.20** was carried out by using either $NaNO_2$[106] or isoamyl nitrite[107] giving 56% and quantitative yields respectively.

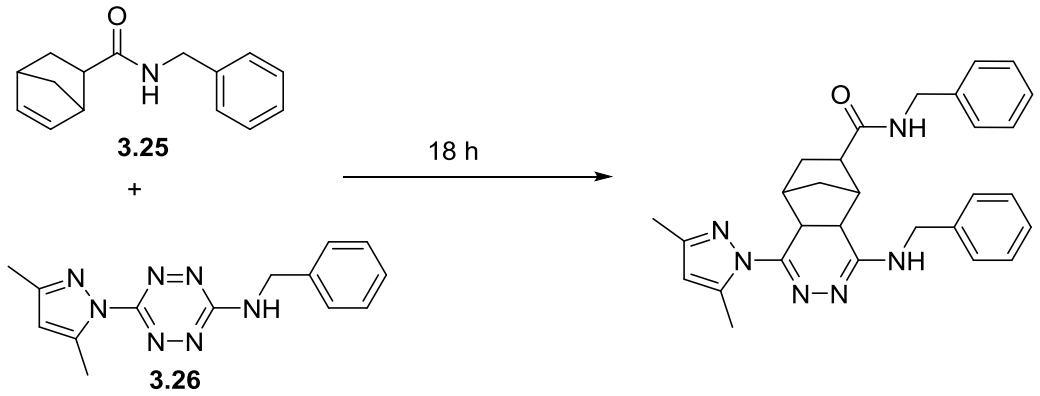


Scheme 3.10: Synthesis of dichloro-tetrazine **3.17** from guanidine hydrochloride **3.21**. Reaction in the box done by Elizabeth Jameson.

The dimethylpyrazol-TZ **3.20** was first coupled to the *N*-terminus of the PNA (using the same conditions as for the dichloro tetrazine **3.17**), and then reacted under

the same conditions as in **Scheme 3.9** with the peptide **3.18**. Unfortunately, this did not result in the formation of any desired product. For a better understanding of the Diels–Alder reaction and to optimise the conditions for the desired product, two small molecules were synthesised to mimic this reaction (**Table 3.1**).

Table 3.1: Reverse electron demanding Diels–Alder cycloaddition test reactions between **3.25** and **3.26**.



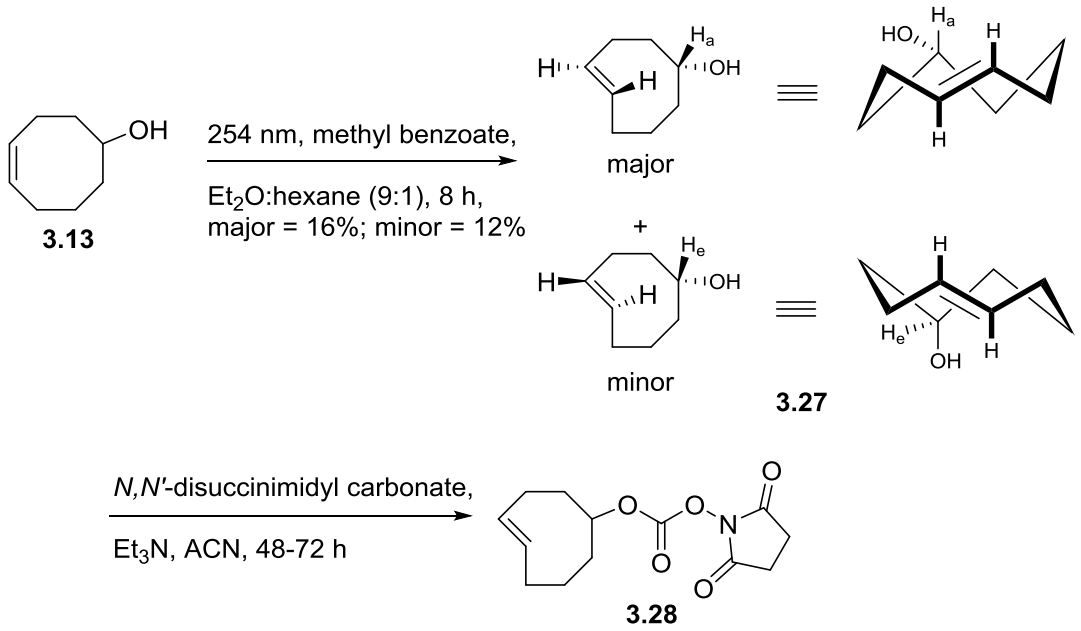
	1 eq of alkene 3.25			*3 eq of alkene 3.25*		
Temp ˚C	***DMSO***	***NMP***	***DMF***	***DMSO***	***NMP***	***DMF***
RT	0%	0%	0%	0%	0%	0%
45	6%	3%	6%	4%	3%	3%
110	42%	30%	38%	27%	38%	33%

Area of product peak under the ELSD-HPLC chromatogram given in %

Table 3.1 shows the different reaction conditions used for this ligation test reaction, including alterations in the equivalence of alkene **3.25** to tetrazine **3.26**, reaction temperatures and solvents. From this it could be concluded that a higher reaction temperature than 110 ˚C or longer reaction time than 24 h would be needed to give a fully complete reaction. Therefore a different TZ/alkene pair was investigated.

3.4.2. Synthesis and test ligation of trans*-cyclooctane*

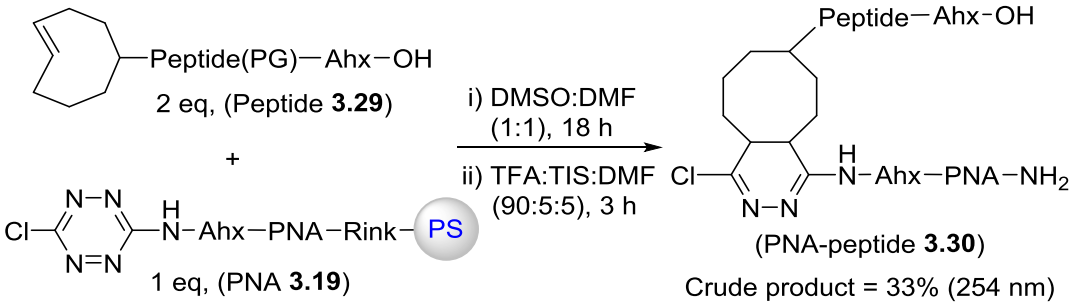
Parallel to the synthesis of the tetrazine moiety, the alkene moiety was also investigated. It has been shown that *trans*-cyclooctane rapidly undergoes the cycloaddition with a TZ.[83] The *cis*-cyclooctanol **3.13** can be prepared from 1,5-cyclooctadiene **3.11** in two steps as described in **Scheme 3.6**. Here the *cis*-isomer was used to make the *trans*-isomer **3.27**.



Scheme 3.11: Synthesis of *trans*-cyclooctene succinimide carbonate **3.28** starting from previously prepared *cis*-cyclooctenol **3.13**.

The reaction involved *cis–trans* isomerisation using UV light at 254 nm in the presence of methyl benzoate. This was left for 12 h and at every 2 h interval the reaction solution was passed through a column containing $AgNO_3$ on silica. $AgNO_3$ binds strongly to *trans*-cyclooctane **3.27** but not to the *cis*-isomer **3.13**, therefore removing the product from the reaction. The yield of this reaction was low, and the two isomers of the product were collected in 16% and 12% yields. Attempts to scale-up this reaction by constantly pumping the solution through $AgNO_3$ on silica and manual filtering out the product failed.

The *trans*-cyclooctanol was coupled to the peptide by first converting the alcohol to the succinimide carbonate **3.28** (**Scheme 3.11**), and then reacting for 18 h with the free *N*-terminus of the model peptide. After cleavage from the resin, peptide **3.29** was reacted with the prepared chlorotetrazine–PNA **3.19** (on solid support) for 18 h (**Scheme 3.12**). After work up, the analysis showed the formation of the PNA–peptide **3.30**, but yet again showed the presence of unreacted starting PNA. This may have been due to the re-isomerisation of some of the *trans*- to the *cis*-cyclooctane, which is less/non-reactive,[83] or it could also be steric hindrance as both of the fragments are large molecules.

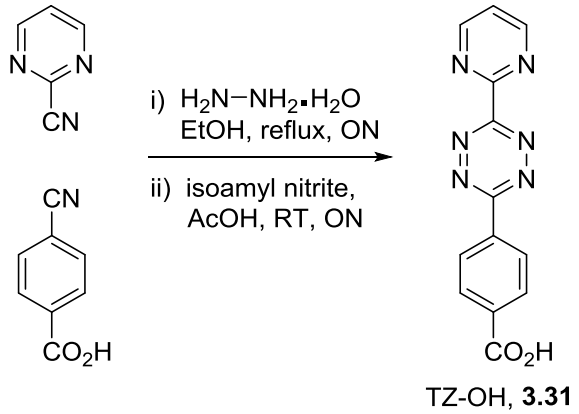


Scheme 3.12: Ligation test using *trans*-cyclooctene–peptide **3.29** and Cl-TZ-PNA **3.19**.

3.4.3. Mono-Acid tetrazine

Following on from the difficulties with the Diels–Alder ligations, a different strategy was employed. 5-Norbornene-2-carboxylic acid was used, as this has been used in the literature in conjunction with 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid **3.31** (TZ-OH).[107] It is reported that the Diels–Alder reaction between those two moieties takes place within 10 min.[85] Synthesis of the tetrazine **3.31**, following a procedure from a patent,[108] proved to be difficult. The maximum yield obtained was 5% using the published purification methods, but some symmetrical acid by-product was still present. The same group published a paper with an altered method, which improved the yield, but still resulted in the symmetrical acid contamination after

purification.[107] To overcome this, the product was purified by flash column chromatography, resulting in the desired pure product in 13% yield (**Scheme 3.13**). This low yield could have been because of the product is very polar and even using 20% methanol and 5% AcOH in DCM, it migrated very slowly down the silica column.

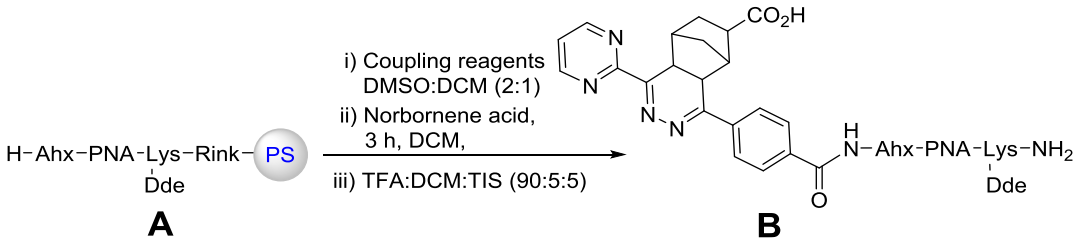


Scheme 3.13: Synthesis of 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid **3.31**.

To a solution of the TZ-OH **3.31** in methanol, one equivalent of the norbornene was added. The completion of the reaction could be followed/estimated visually, as the tetrazine solution was purple/red, which turned colourless/yellow once the reaction was completed within 30 min.

Next, different reaction conditions for the coupling of the TZ on solid phase were screened with product formation analysed by HPLC and MALDI-TOF MS. **Table 3.2** displays the results of coupling tests with the first column showing the different activation reagents used. For all the experiments the TZ was dissolved in a mixture of dimethyl sulfoxide (DMSO):DCM. It was noted that under the cleavage conditions (TFA:TIS:DCM, 90:5:5) that the TZ functionality was decomposing. To analyse which coupling method worked best, norbornene acid was added to the TZ before the cleavage of the test PNA. From this screening test, DIC/Oxyma and *N*-hydroxysuccinimide (HOSu)/py coupling protocols were selected to further test and optimise the reaction conditions.

Table 3.2: TZ peptide coupling screening.

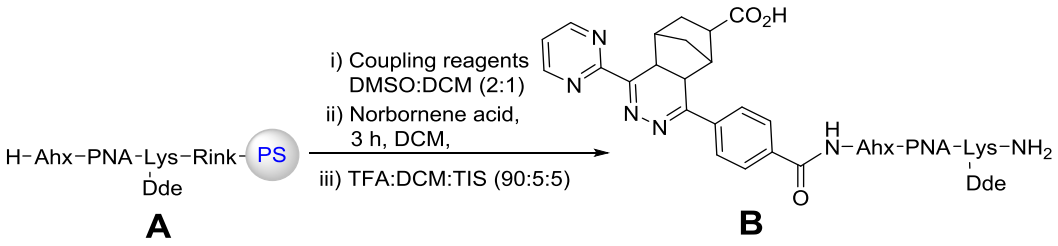


	1 h, 60 ˚C		*3 h*		*24 h*	
DIC	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗
	ELSD:	A: 55% B: 0%	**ELSD:**	A: 70% B: 0%	**ELSD:**	A: 62% B: 0%
DIC Oxyma	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓
	ELSD:	A: 77% B: 5%	**ELSD:**	A: 90% B: 0%	**ELSD:**	A: 32% B: 12%
HATU DIPEA	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✗
	ELSD:	A: 83% B: 0%	**ELSD:**	A: 91% B: 0%	**ELSD:**	A: 40% B: 0%
HBTU DIPEA	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓
	ELSD:	A: 55% B: 0%	**ELSD:**	A: 44% B: 0%	**ELSD:**	A: 20% B: 0%
HCTU DIPEA	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗
	ELSD:	A: 65% B: 0%	**ELSD:**	A: 81% B: 0%	**ELSD:**	A: 79% B: 0%
EEDQ	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗	**MS:**	A: ✓ B: ✗
	ELSD:	A: 56% B: 0%	**ELSD:**	A: 71% B: 0%	**ELSD:**	A: 82% B: 0%
EDC HOSu py	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓	**MS:**	A: ✓ B: ✓
	ELSD:	A: 30% B: 36%	**ELSD:**	A: 36% B: 35%	**ELSD:**	A: 45% B: 22%

MS: ✗ indicates that the mass of the product ***B*** or starting material ***A*** is not seen and the ✓ indicates the presence of the correct mass; **ELSD**: gives the relative % according to the area of the peak in the HPLC chromatogram.

The different optimisation reaction conditions can be seen in **Table 3.3**. The tetrazine acid (TZ-OH, **3.31**) was coupled using Oxyma and DIC with different reaction times, from 1 h to 24 h in DMF. From these results, it can be seen that the tetrazine decomposed when left overnight and no difference was observed between a 2 × 1 h and the 2 × 3 h coupling. It was noted that a solution Oxyma, DIC and tetrazine in DMF was colourless after 18 h, indicating decomposition in DMF. However, this was not seen when DMSO was used. As for the succinamide tetrazine, different solvent mixtures were used for the coupling (**Table 3.3**). The best result was obtained when a mixture of DMF/pyridine (py) (19:1) was used, and in this mixture the tetrazine did not decompose.

Table 3.3: TZ peptide coupling optimisation.



TZ-OH, Oxyma/DIC			*TZ-OH, HOSu/py, 18 h*			
2 × 1 h	***2 × 3 h***	***3 h + 24 h***	***DMSO/ DCM (2:1)***	***DMSO/ Py (19:1)***	***DMF/ Py (19:1)***	***DCM***
77%	77%	37%	78%	80%	88%	24%

% based on area in HPLC chromatogram with an ELS detector

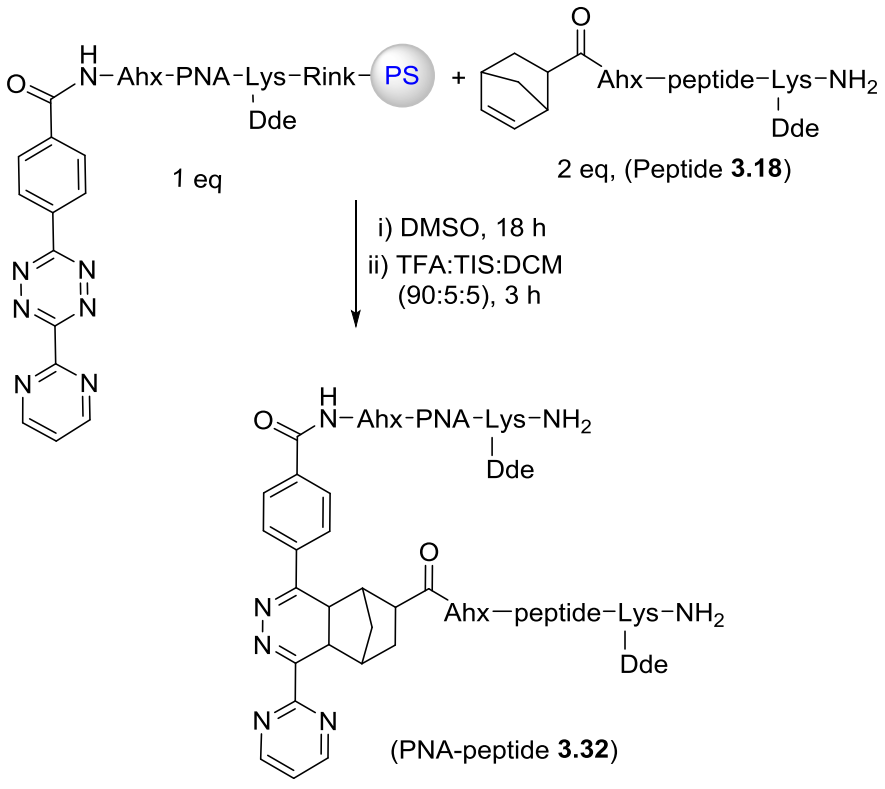
Finally, the coupling conditions of the two best results were repeated, to evaluate which would be the best for the synthesis of the library (**Table 3.4**). Using Oxyma/DIC in DMSO/DCM (2:1) for 2 × 1 h showed the best results.

Table 3.4: Re-evaluation of optimised TZ peptide coupling reactions.

TZ-OH, Oxyma/DIC, 2 × 1 h DMSO/DCM	*TZ-OH, HOSu)/py, 18 h, DMF/Py*
85%	***31%***

% based on area in HPLC chromatogram with an ELS detector

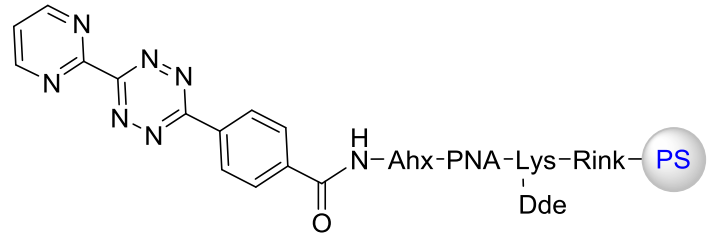
To test the ligation with this tetrazine, the norbornene functionalised peptide **3.18** was added to the TZ-PNA resin (**Scheme 3.14**). Following the conditions used before for other ligation methods, the conjugate **3.32** was cleaved and analysed. Again, there was still some of the unreacted TZ-PNA present (30%), so it was decided to attempt the ligation in solution.



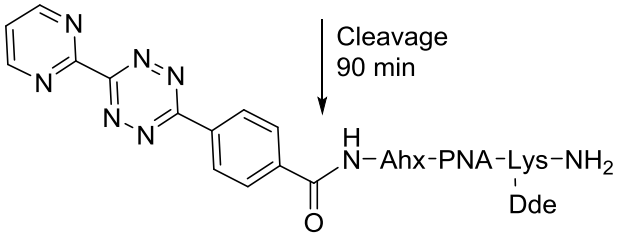
Scheme 3.14: Ligation test using norbornene-peptide **3.18** and TZ-PNA.

For the ligation in solution the TZ would need to remain intact after cleavage. From some of the earlier cleavages when the standard cleavage mixture was used (TFA:TIS:DCM, 90:5:5) the red colour of the tetrazine disappeared after about two hours, probably due to the tetrazine being reduced. Different cleavage mixtures were screened and the reactions were analysed by ELSD-HPLC and MALDI-TOF MS (**Table 3.5**, **Figure 3.2a**).

Table 3.5: TZ-PNA of Rink linker cleavage condition screening.



TZ-PNA Resin **3.34**



TZ-PNA **3.33**

A: ***TFA:TIS:DCM*** *(90:5:5)*	***B:*** ***TFA:TIS:DCM*** *(45:5:50)*	***C:*** ***TFA:TIS:DCM:PhOH*** *(88:2:5:5)*	***D:*** ***TFA:TIS:DCM:PhOH*** *(44:1:52.5:2.5)*
75%	*88%*	*84%*	*83%*
E: ***TFA:PhOH*** *(95:5)*	***F:*** ***TFA:H_2O*** *(95:5)*	***G:*** ***TFA:PhOH:$MeSO_3H$*** *(90:5:5) 2 × 15 min*	***H:*** ***TFA:DCM:Indole*** *(70:28:2)*
87%	*96%*	*42%*	*20%*

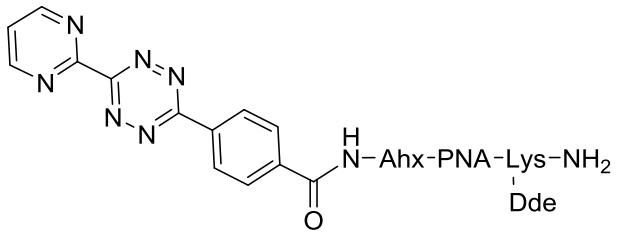
% based on area in HPLC chromatogram with an ELS detector

From the **Table 3.5** it can be clearly seen that the best conditions for cleaving the TZ-PNA **3.33** was achieved when TFA:H_2O (95:5) was used. As for the others,

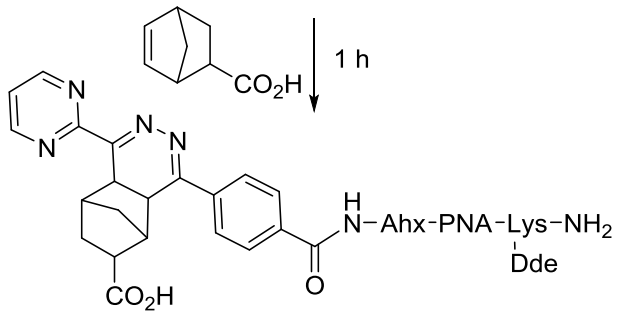
PhOH as a scavenger also gave a relatively good result. The lowest yield was obtained in the case were indole was used in the reaction. It is known that indole can also act as a dienophile in the presence of TZ, and hence the yield for the desired product may have been reduced.[109]

As a final test to determine if the TZ was still active towards the ligation after the cleavage, 5-norbornene-2-carboxylic acid was added to the crude **3.33** fragment and analysed (**Table 3.6**, **Figure 3.2b**). As a control, norbornene acid was ligated to a TZ-PNA resin **3.34** before cleaving the Diels–Alder product from the Rink-liner using the standard methods (TFA:TIS:DCM, 90:5:5). This resulted in a crude purity of **3.35** of 88%. The best result for the Diels–Alder reaction in solution was obtained when the TZ as cleavage with TFA and water in a 95:5 ratio. This showed that the TZ was still active in the Diels-Alder reaction, following the TFA treatment.

Table 3.6: Crude TZ-PNA **3.34** from **Table 3.5** treated with Norbornene.



TZ-PNA **3.33**



3.35

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
45%	*46%*	*40%*	*51%*	*45%*	*58%*	*16%*	*0%*

% based on area in HPLC chromatogram with an ELS detector

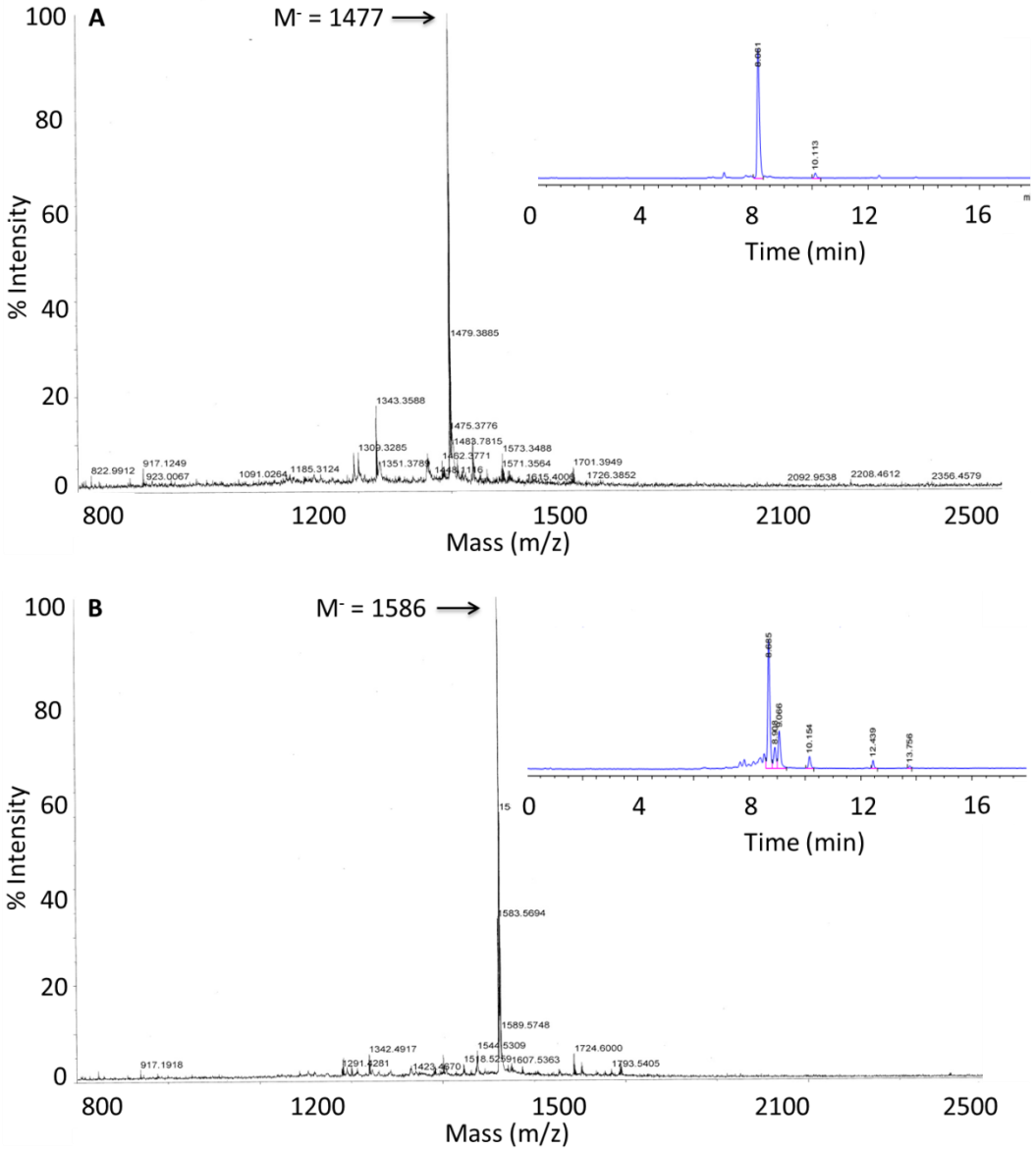


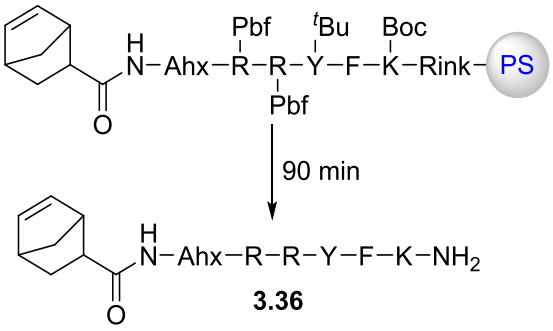
Figure 3.2: ***A)*** HPLC-ELSD and MALDI-TOF MS after cleavage using TFA:H_2O (95:5) TZ-PNA **3.33**; and ***B)*** HPLC-ELSD and MALDI-TOF MS of reaction between norbornene and TZ-PNA **3.33** to form the conjugate **3.35**.

3.4.4. Stability of norbornene during TFA cleavage

In a paper published by Brown,[110] it was suggested that TFA can add to the double bond of norbornene even at low temperatures. As the cleavage conditions for peptides linked to a resin functionalised with the Rink-linker involve high

concentrations of TFA, and most of the peptides that have been used in the study were synthesised with an acid sensitive chloro trityl linker, a study was undertaken of the stability of norbornene. For this purpose, a small peptide with different functionalities (arginine (Arg), Lys, phenylalanine (Phe) and Tyr) and protecting groups (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), Boc and ^{t}Bu) was synthesised on an amino-methyl polystyrene resin with a Rink linker. After the coupling of 5-norbornene-2-carboxylic acid using Oxyma and DIC as activators, the peptide was cleaved under different conditions and analysed (**Table 3.7**, **Figure 3.3a**). None of the cleaved products showed the addition of TFA across the norbornene double bond. Some of the Pbf protecting group was not removed under the conditions used, and a longer cleavage time was needed for complete deprotection. This though was not a problem as the norbornene was stable under vigorous TFA cleavage conditions.

Table 3.7: Norbornene test cleavage.



3.36

*TFA:TIS:DCM (90:5:5) **2.31***	*TFA:TIS:DCM (90:5:5)*	*TFA:TIS:DCM (45:5:50)*	*HFIP:HCl[a]:DCM (88:10:2)*	*HFIP:HCl[a]:DCM (98:1:1)*
100%[b]	*100%*	*100%*	*79%*	*100%[c]*

% based on area in HPLC chromatogram with an ELSD; [a] conc. HCl; [b] TZ-OH **3.31** was added before cleavage as a positive control; [c] Pbf protection group still attached.

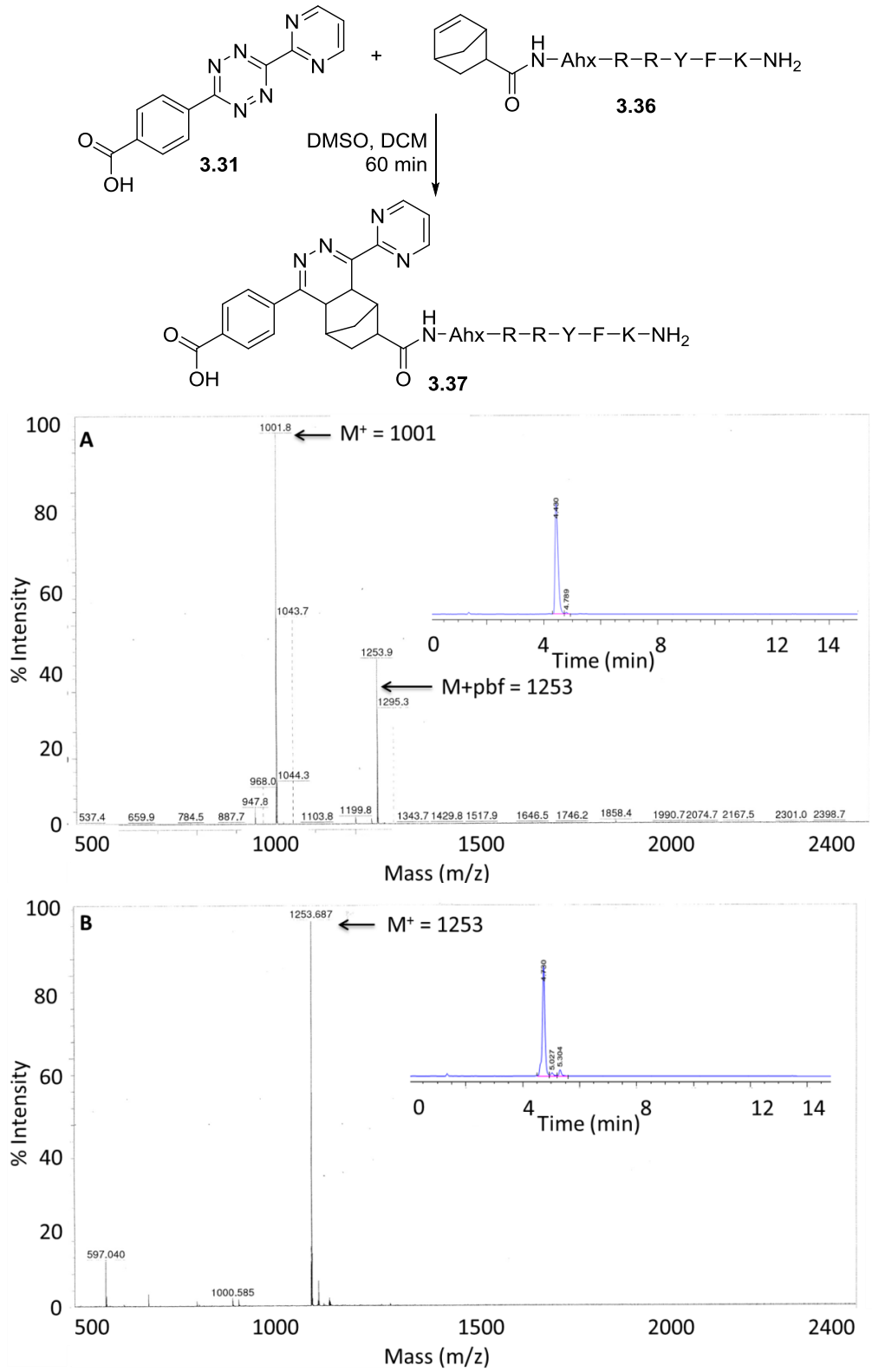


Figure 3.3: Peptide **3.36** reaction with TZ-OH **3.31** after peptide cleaved from resin using TFA: **A)** MALDI-TOF MS and HPLC of starting peptide **3.36**; **B)** MALDI-TOF MS and HPLC of conjugate **3.37**.

A solution of TZ-OH **3.31** in DMSO and DCM (1:1) was added to the cleaved peptide. After 60 min reactions were analysed by HPLC and MALDI-TOF MS (**Figure 3.3 b**), and they all showed the conversion of the starting material **3.36** to the desired product **3.37**. For peptides where the Pbf group was not deprotected, the mass of the adduct **3.37** plus the Pbf-groups was seen in the MALDI-TOF MS. As a control, peptide **3.36** was also made without the norbornene functionality. This peptide was then treated with the same TZ-OH solution. Upon analysis by LC-MS and HPLC-ELSD, the TZ-OH signal had disappeared. This was an unexpected result. TZ-OH **3.31** is unstable under basic environments, but in the stock solution (**3.31** in DMSO:DCM (1:1) with 0.1% DIPEA) the TZ-OH was stable for several days. This would perhaps indicate that the arginines in the peptides are basic enough to decompose the tetrazine.

3.5. Conclusion

In conclusion, the best ligation method was the reaction between 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid **3.31** and 5-norbornene-2-carboxylic acid. The coupling reaction of the tetrazine to a resin bound amine was found to be ideal when using Oxyma/DIC in DMSO/DCM (2:1) for 2 × 1 h, while the cleavage conditions were best using TFA:H_2O (95:5). It was also shown that norbornene was stable under the standard TFA cleavage conditions, which is in contrast to work published by Brown *et al.*.[110]

Chapter IV

Switching on "FISH-probes" – in situ PNA hybridisation followed by fluorophore ligation

4. Evaluation of a PNA *in situ* hybridisation and fluorophore ligation strategy

4.1. Introduction

Fluorescent *in situ* hybridisation (FISH) is a cytogenetic technique that visualises the presence of specific DNA/RNA sequences and their location, be it in the nuclei or the cytoplasm. FISH probes are synthesised with a complimentary sequence to the target sequence and with a fluorophore attached. This probe hybridises to the target DNA/RNA sequence (**Figure 4.1A**) and can be analysed by fluorescence microscopy. **Figure 4.1B** and **C** shows FISH being used to show the presence of a specific DNA sequence on a chromosome (red dots), and mRNA inside the cytoplasm (white dots).

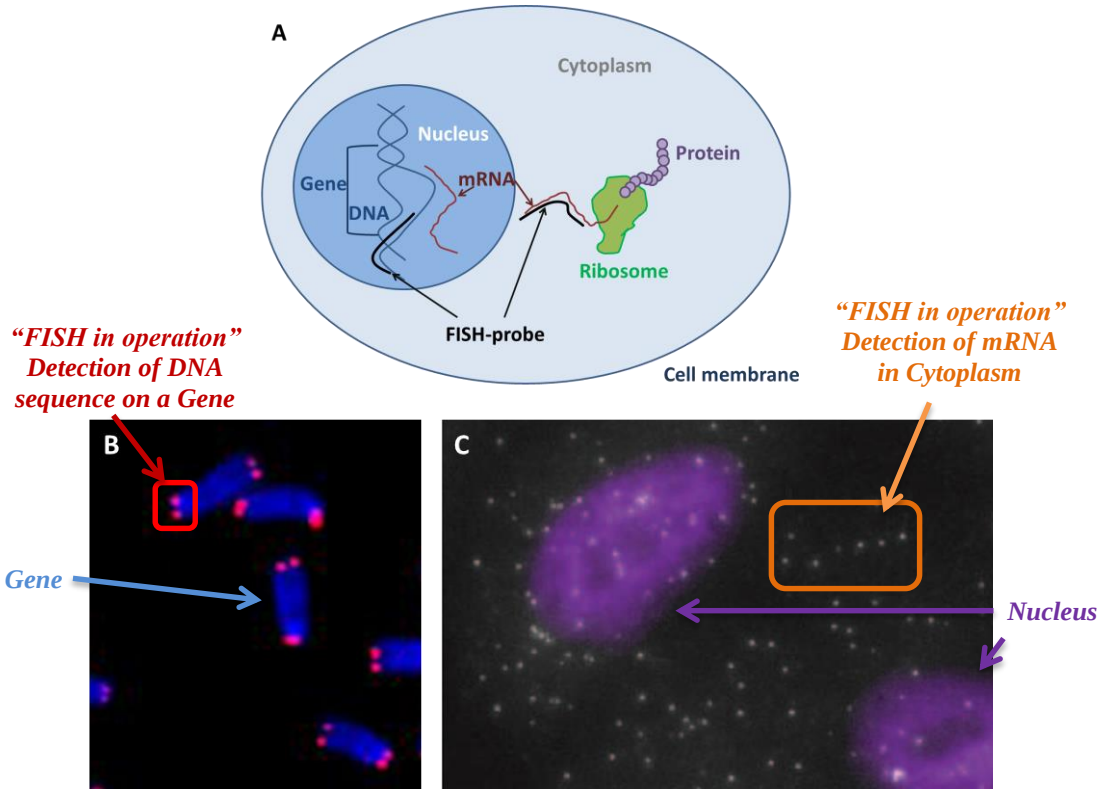


Figure 4.1: A: Cell with a FISH-probe hybridising to a DNA or RNA target; **B:** Image of FISH used to highlight in red DNA sequences on a chromosomes; **C:** Image of FISH being used to show mRNA sequences inside the cytoplasm (dots).[111] (Image **B** as published by Genet[112] in the open access journal Molecular Cytogenetics, and **C** as published by Shaffer[111] in the open access journal PLoS ONE).

FISH developed from *in situ* hybridisation methods which were first reported in the late 60's by John[113] and Pardue[114] using radiolabelled RNA/DNA probes. To improve signal detection, reduce the time for imaging and increase the safety aspect, various methods were developed that used fluorescence instead of radiolabels.[115,116] This included the use of biotinylated DNA probes that could be detected by either labelled streptavidin,[117] or with a rabbit anti-biotin antibody, followed by a fluorescein-labelled anti-rabbit antibody.[118] FISH is used in clinical situations for the detection of mutations related to illnesses, personalised medicine, and for the detection and classification of hematopoietic malignancy (leukaemia, multiple myeloma, amongst others) and non-hematopoietic malignancy (lung cancer, breast cancer, amongst others).[119] Following the development of PNA,[24] *in situ* hybridisation with PNA was utilised for FISH experiments, with advantages over DNA probes due to stronger affinities towards target DNAs in comparison to DNA-DNA interactions.[120]

Taneja[120] compared a DNA (30mer) probe labelled with fluorescein and a PNA (15mer) probe labelled with Cy3, to analyse the presence of triple repeats (CTG) in DNA in fixed human myotonic dystrophy cells. In this experiment, a DNA-probe was firstly hybridised, followed by addition of a PNA-probe, which targets the same DNA-target as the DNA-probe. The PNA-probe was found to completely replace the DNA-probe. In addition, it was observed that the PNA-probe identified more targets in the sample compared to the DNA-probe, and the PNA-probe florescence signal was more intense with respect to the DNA counterpart probe. Both of these indicate that the PNA-probe was more selective and has a higher affinity for the DNA-target compared to the larger DNA-probe. Santangelo[121] used molecular beacons (Cy3/black hole quencher 2 (BHQ-2) / Cy5/BHQ-3) to visualise real-time dynamic measurement of RNA localisation in living cells with response to drug molecules or external stimulants. FISH was used (which usually uses fixed cells) to confirm the RNA localisation pattern in live cells, which showed good correlation with respect to the live cells.

Another development using the principle of FISH was termed "small molecule FISH" (smFISH) by Femino.[122] Femino used five oligonucleotide probes

that hybridise to adjacent sequences on mRNA to determine the target mRNA molecule location inside a cell. Several different mRNA targets have been tested. In all, it was demonstrated that using this approach of multiple dye molecules on a single probe improved the sensitivity and increased the signal to noise ratio when compared with a single probe labelled with one dye. The oligomers were between 50 and 65 nucleobases, and each molecular probe had five fluorophore molecules attached (**Figure 4.2 A**). Many examples exist in which smFISH was applied.[111,123,124] Tyagi[125,126] attached only one dye to a 3' position of the probe, but used multiple probes (50) that bind sequentially to the target RNA. One of their examples was the visualisation of the mRNA of the fusion protein BCL–ABL. Two sets of probes were synthesised that detected the exon 1 sequence located at the 5' end of BCR mRNA and the exon 11 of ABL mRNA that is located near the 3' end (**Figure 4.2 B**).

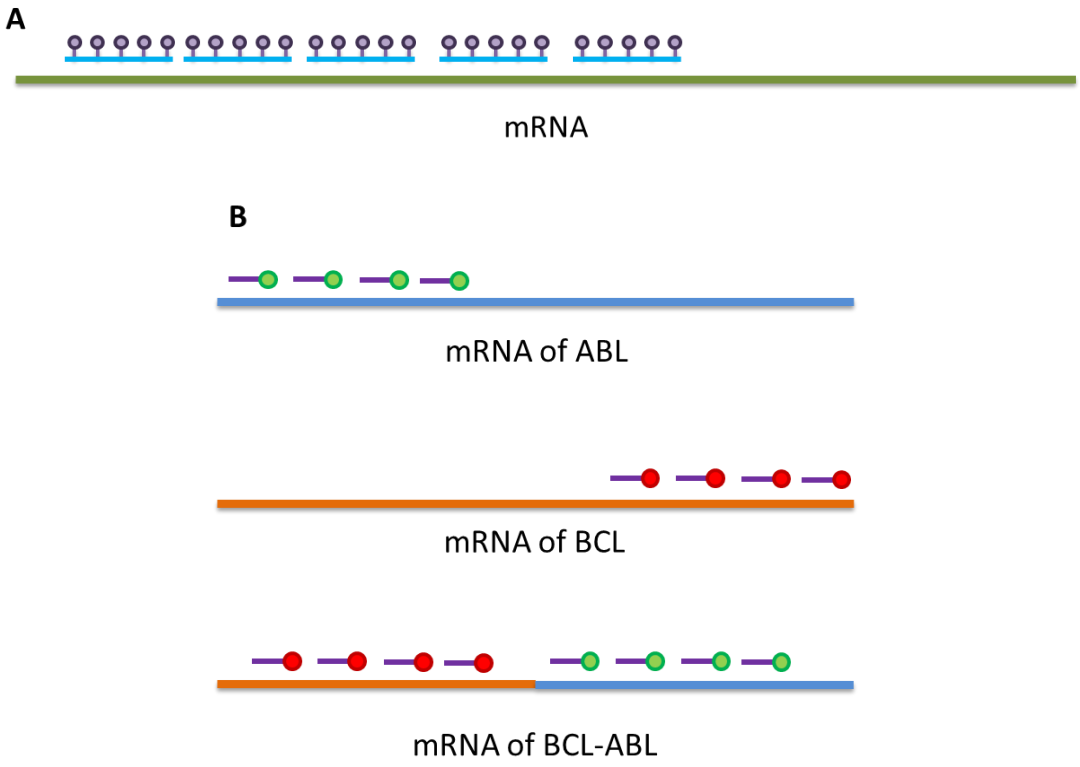


Figure 4.2: A: Five smFISH probes with five fluorophore per probe used by Femino;[122] **B:** smFISH method used by Tyagi to detect the mRNA fusion BCL-ABL.[126]

The probes were labelled with TAMRA (for ABL) and Alexa Fluor 594 (for BCR). In the smFISH experiment, K562 cells were used as they express the target fusion protein, and HeLa (cervical cancer) cells were used as a negative control. The individual signals for both ABL and BCR where visible in both cell lines, but only in K562 cells both of the signals (ABL and BCR) were located in the same spot (**Figure 4.3**), indicating the percent of BCL–ABL fusion mRNA.[126]

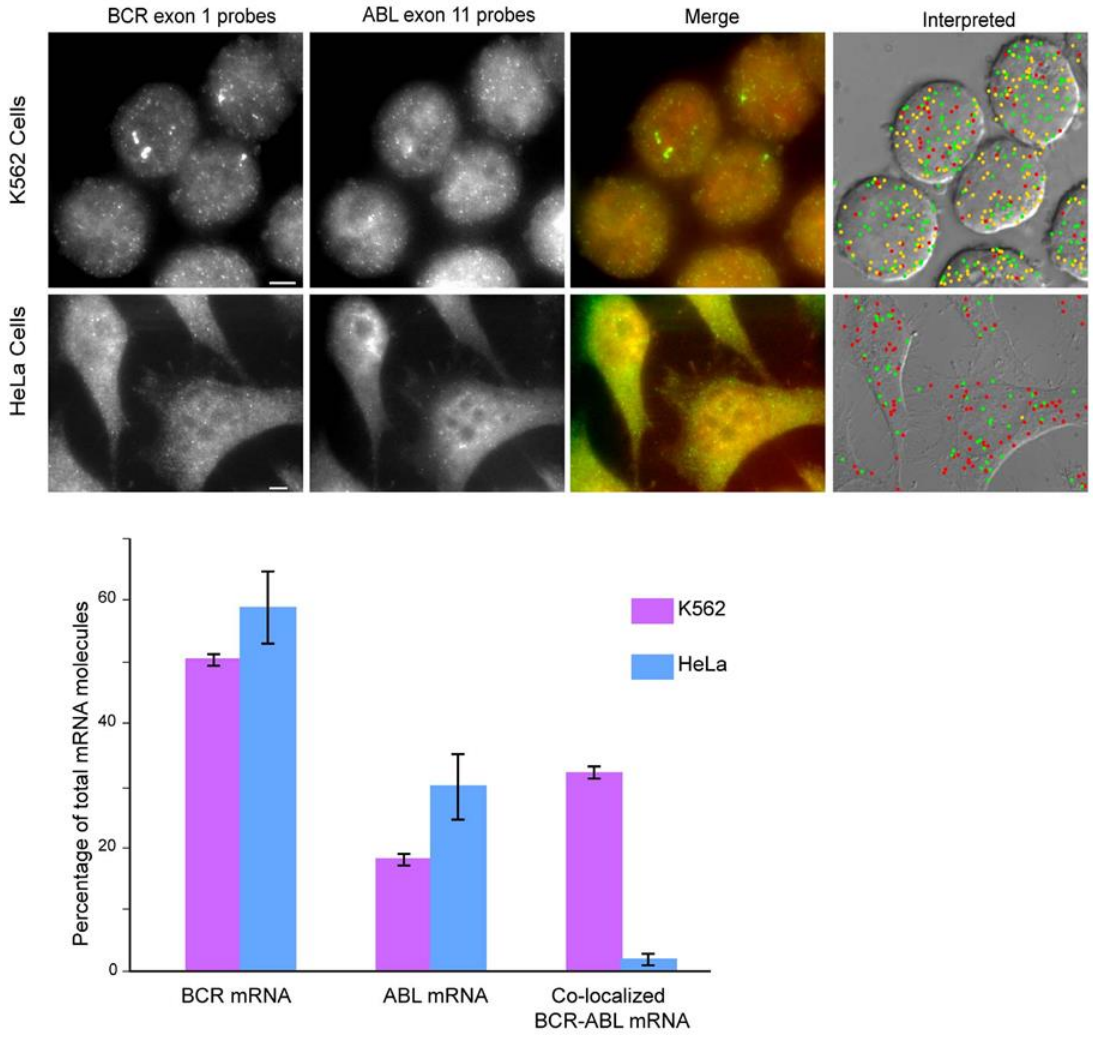


Figure 4.3: Top: Cell images of K562 images showing the presence of ABL (red dots), BCR (green dots) and BCR-ABL (yellow dots) mRNA, and HeLa cells (negative control) only showing the presence of ABL and BCR (as published by Markey[126] in open access journal PLoS ONE).

Another method for intracellular detection of DNA strands is based on the azide–alkyne Huisgen cycloaddition (**Figure 4.4**).[127] A modified nucleobase was

introduced to a cell and during cellular replication and incorporated into the DNA strand. An advantage of using these small nucleobase modifications (alkyne or azide functionalised) compared to others, where the functional group is a fluorophore, is that the smaller monomers do not interfere significantly with the folding and hydrogen bonding of the DNA duplex.[128] Many examples exist in the literature where such monomers are introduced into DNA followed by the ligation of a fluorophore using the copper(I)-catalysed azide–alkyne cycloaddition (CuAAC).[129–132]

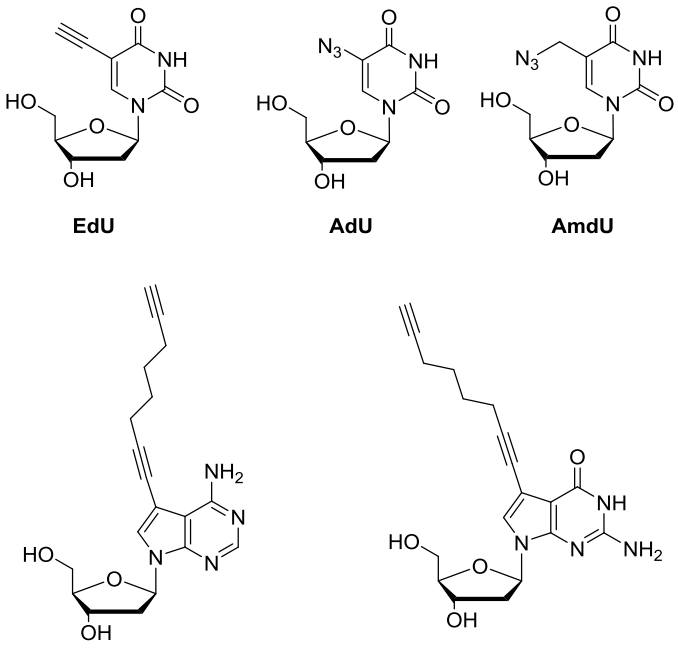
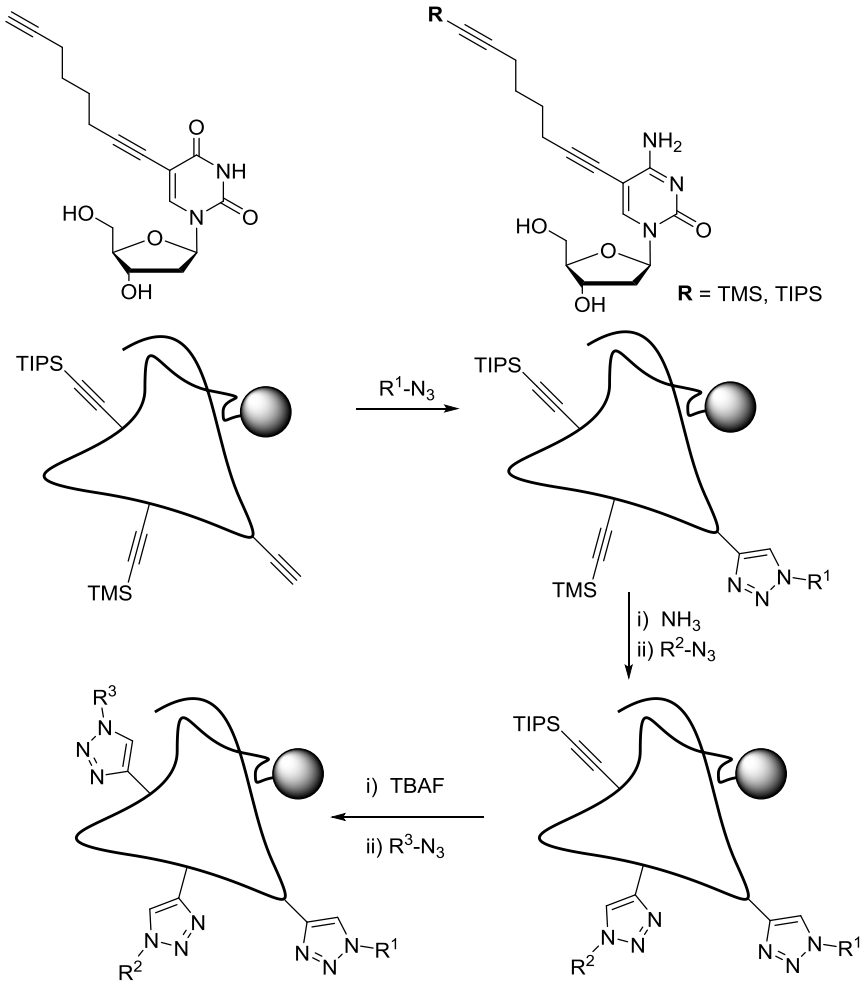


Figure 4.4: Some examples of modified bases incorporated into DNA and subsequently modified using a CuAAC coupling reactions.

In order to address the toxicity issue of copper(I) and its ability to partially degrade DNA and proteins,[103,133] Luedtke[132] investigated the use of a copper free Huisgen cycloaddition, known as "strain-promoted AAC" (spAAC). In the experiment the group was comparing the ligation effectiveness of both the CuAAC and spAAC, which showed that spAAC was not as effective in the ligation when the DNA was not denatured before the ligation. When the DNA was denatured the fluorescence intensity was the same for both reactions. Interestingly, this experiment

showed evidence that the copper free cycloaddition ligated to single stranded DNA, whereas CuAAC ligates throughout the DNA sequence. This differentiation is useful in identifying single stranded DNA in the nucleus.

Another interesting development using the CuAAC reaction was carried out by Carell,[69] who used three different alkyne nuclear base monomers, and introduced them into DNA. Two of the monomers had a protecting group that can be orthogonally removed, freeing the alkyne that can then undergo CuAAC with different azides. Here three different labels where coupled on to the DNA (**Scheme 4.1**), including biotin and several different fluorophores were introduced.



Scheme 4.1: Top: Modified nucleobases used for the synthesis in the DNA synthesis; **and Bottom:** Labeling of the DNA strand with three different probes using CuAAC reaction. After the first click reaction, the TMS protection group was removed, followed by the second click reaction. TIPS was then removed subsequently before the third click reaction.

4.2. Results and Discussion

The approach here was to utilise tetrazine chemistry to label PNA-probes. Thus TZ-PNA was hybridised *in situ* to a mRNA target, followed by *in situ* ligation of a fluorophore (**Figure 4.5**).

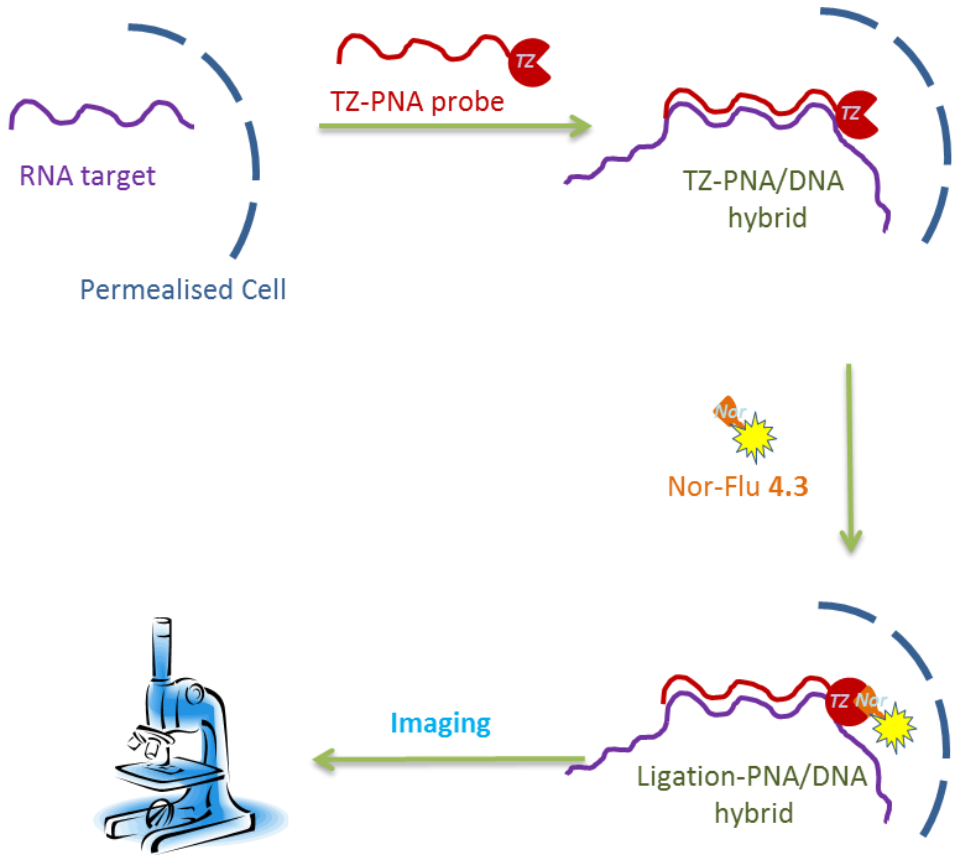


Figure 4.5: FISH experimental procedure: After fixing the cells the TZ-PNA probe was hybridised onto the RNA target, followed by ligation of Nor-Flu **4.3** to the tetrazine, followed by washing and imaging of the cells by microscopy.

PNA probes targeting the mRNA of glyceraldehyde 3-phospate dehydrogenase (GAPDH) and Ribosomal RNA 28S were used to verify the approach in fixed HeLa cells (**Figure 4.6**).

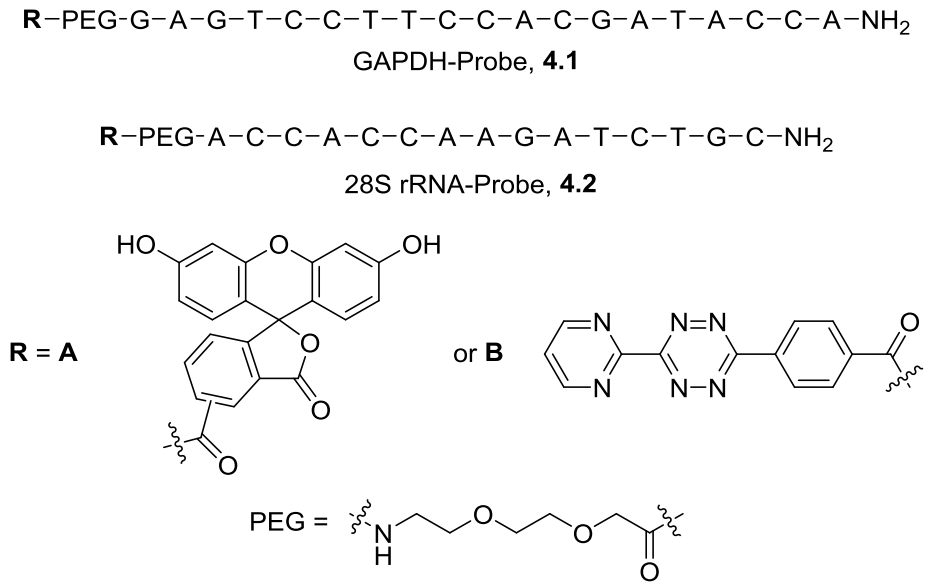
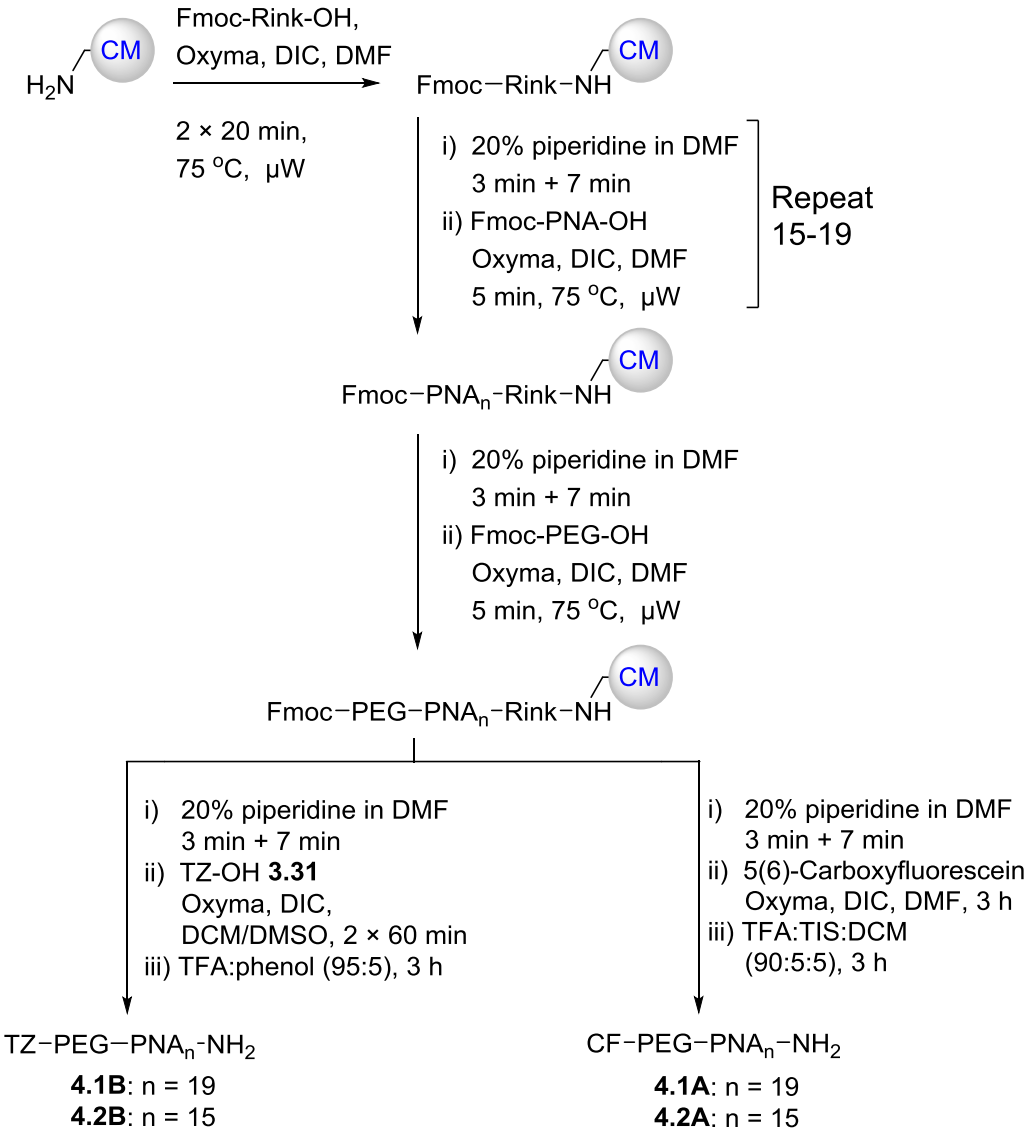


Figure 4.6: PNA probes used for the FISH experiment.

FISH experiments were carried out with linear PNAs, which were functionalised with TZ-OH **3.31**. Detection of TZ probes was carried out by intracellular hybridisation to the target RNA followed by incubation with norbornene functionalised 5-aminofluorescein **4.3** (see **Figure 4.5**, and **Scheme 4.3**). This system has the advantages of a short PNA sequences being used unlike the approach of Santangelo,[121] which simplifies the synthetic procedure and purification. A carboxyl fluorescein-PNA (CF-PNA) construct was used as a positive control to validate the hybridisation of the probe to the target mRNA inside cells.

4.2.1. PNA synthesis

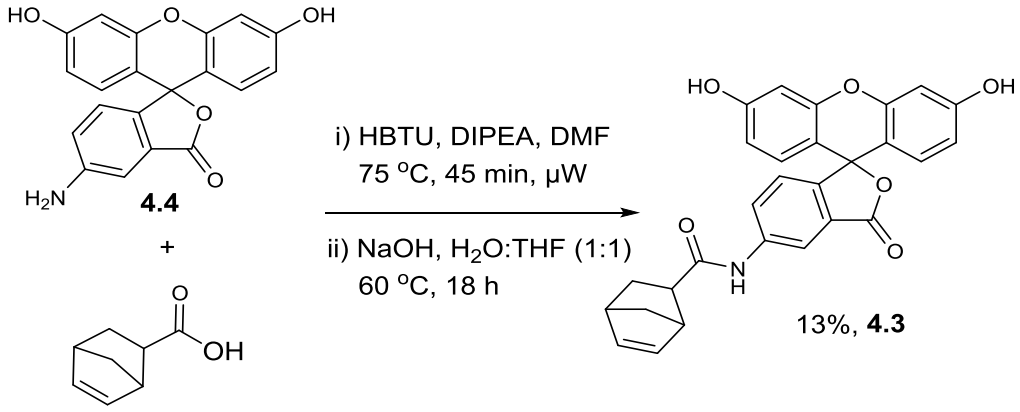
Synthesis of the PNA probes was carried out using a semi-automated peptide synthesiser with microwave heating on ChemMatrix resin with an attached Rink-amide linker. Following PNA preparation (**Figure 4.6**), TZ-OH **3.31** or 5,6-carboxyfluorescein were coupled to the PNAs (**Scheme 4.2**).



Scheme 4.2: Synthesis of PNA probes **4.1A**, **4.1B**, **4.2A**, and **4.2B** using a Biotage® Initiator+ SP Wave peptide synthesiser with ChemMatrix (CM) resin.

Employing cleavage conditions optimised for tetrazine functionalised peptides (TFA:H_2O, 95:5; 3 h) resulted in the expected product with the attachment of a group resulting from the cleavage of the Bhoc-protecting groups. Polystyrene resin has a hydrophobic backbone enabling free water molecules to scavenge the released diphenylmethyl carbo-cations. In contrast, the backbone of ChemMatrix-resin is composed of PEG-chains, which may hinder water molecules from scavenging. This effect was avoided by substituting water with phenol, which

resulted in the generation of the expected PNAs. To attach a fluorophore onto the TZ-moiety of the PNA probe by tetrazine ligation, a fluorescent-norbornene conjugate was prepared. This was achieved by coupling 5-aminofluorescein **4.4** to *exo*-5-norbornenecarboxylic acid using HBTU/DIPEA. After purification the desired product **4.3** was obtained in 13% yield.



Scheme 4.3: Synthesis of norbornene-fluorescein **4.3** by coupling norbornene acid to 5-aminofluorescein **4.4**.

4.2.2. In-cell hybridisation and ligation

Fixed cells were incubated with PNA-probes (4 µM) at 37 ˚C for 20 h as described in the literature.[121] The hybridisation solution was removed and the cells were washed, followed by addition of **4.3** (4 µM) to TZ-PNA incubated cells. The ligation was carried out for 2 h and the cells were washed, and stained with a cell nucleus stain 4',6-diamidino-2-phenylindole (DAPI) prior to imaging. As shown in **Figure 4.7** it can clearly be seen that **4.1A** is able to detect GAPDH mRNA by hybridisation (as indicated by strong fluorescence (**Figure 4.7A**)). The use of probe **4.1B** followed by ligation of **4.3** did not result in a significant fluorescence signal (**Figure 4.7B**, shows a very weak signal). This could have been because the ligation had not been completed in the time allocated for the reaction, as intracellular TZ–Nor ligation in fixed cells maybe slower than in solution phase or live cells. A similar result was seen for the 28S rRNA-probe **4.2A** compared with the TZ probe **4.2B** (**Figures 4.7C**

and **4.7D**, respectively). However, the control probe **4.2A** had a remarkably lower fluorescence in comparison to control probe **4.1A**. As the control probe **4.2A** did not show much fluorescence, which indicates that much less 28S mRNA was present in the cell. As a negative control, fixed cells were incubated with the ligation probe Nor-Flu **4.3** in the absence of any PNA and TZ. This was done to show that when cells are treated with **4.3** only, no fluorescence would be observed, and indeed none was observed.

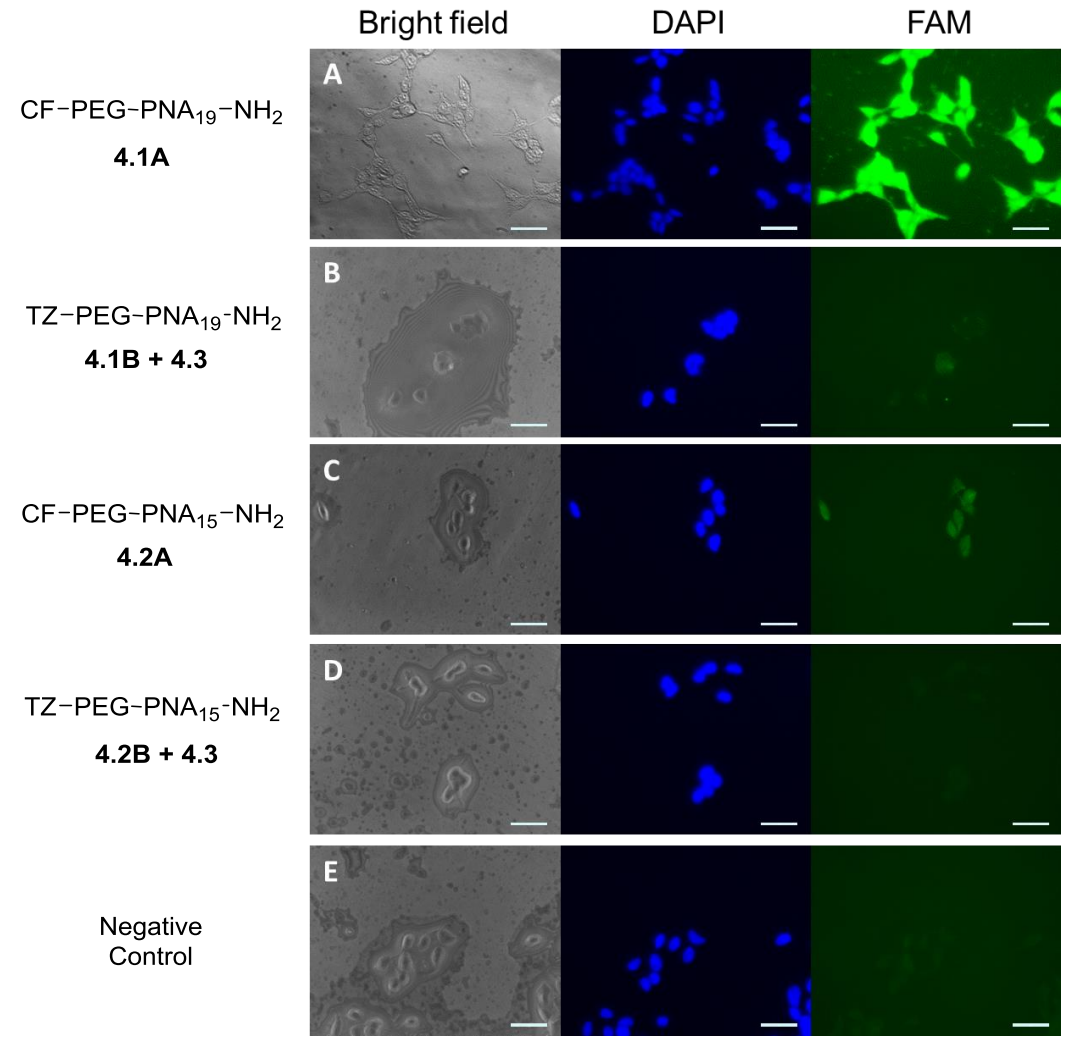


Figure 4.7: FISH experiment using 4 µM of probe **A: 4.1A; B: 4.1B** after incubation with Nor-Flu **4.3** (4 µM)**; C: 4.2A; D: 4.2B** after incubation with Nor-Flu **4.3** (4 µM)**; and E:** cells treated with DAPI and Nor-Flu **4.3** (4 µM). DAPI: λ_{ex} = 340–395 nm; λ_{em} = 430–505 nm; FAM: λ_{ex} = 447–495 nm; λ_{em} = 500–554 nm. Bar = 50 µm

To investigate the intracellular stability of the TZ-norbornene construct, ligation was carried out prior to incubation in the cells and the construct added to cells. It can be seen that the fluorescence intensity increased when the pre-ligated probe was used, (after the ligation, the solution of **4.1B** and **4.3** was treated with TZ-CM resin (prepared in-house) for 2 h to scavenge any unreacted **4.3** before the ligated probe was used in the FISH experiment). As seen in **Figure 4.8B**, the fluorescence increased when the ligation was carried out before the ligation compared to the intracellular ligation, although the intensity was still much lower than the positive control probe **4.1A** (**Figure 4.7A**). This could be an indication that either ligation in fixed cells needs longer reaction times, or that the long-term exposure of the tetrazine to the cellular environment interferes with the TZ chemistry. It is also possible that higher concentrations of the labelled tetrazine are needed to "push" the *in vivo* chemistry to completion. The formaldehyde cellular fixing generates a complex 3D network and this might interfere with the labelling reaction kinetics.

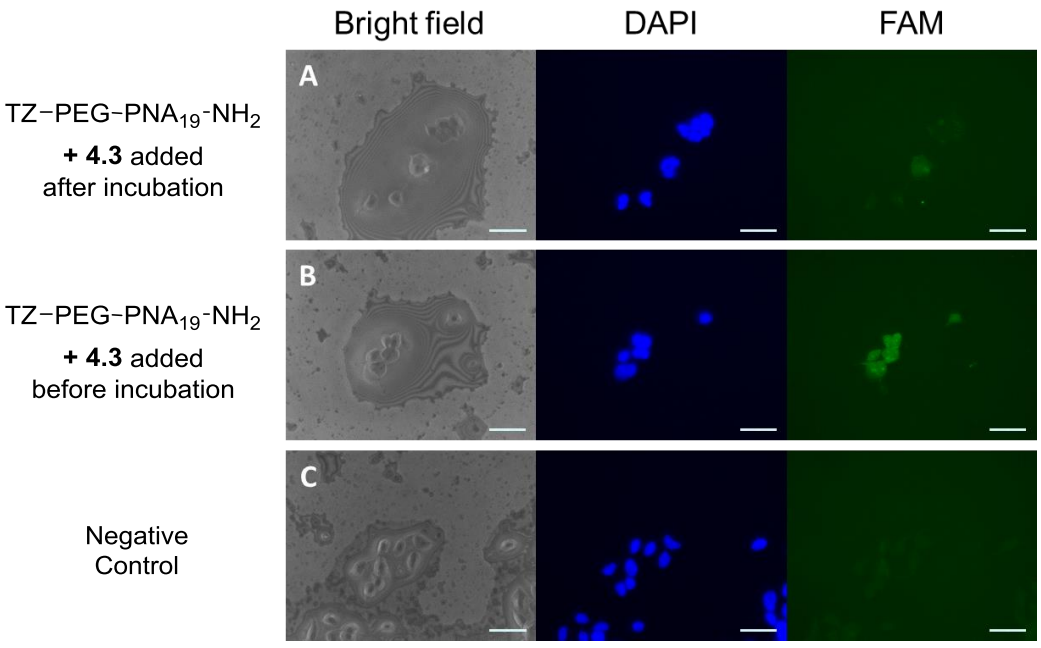


Figure 4.8: A: probe **4.1B** with Nor-Flu **4.3** ligation in cells**; B:** probe **4.1B** with Nor-Flu **4.3** ligation before incubation with cells**; C:** cells treated with DAPI and Nor-Flu **4.3**. DAPI: λ_{ex} = 340-395 nm; λ_{em} = 430-505 nm; FAM: λ_{ex} = 447-495 nm; λ_{em} = 500-554 nm. Bar = 50 µm

4.3. Conclusion

PNAs complementary to two specific mRNA located inside cells were synthesised. The PNAs terminated with either fluorescein or a TZ-moiety was used to ligate a norbornene-fluorescein. However, following hybridisation, the ligation could not be achieved with any great efficiency. Tests of a pre-ligated PNA-fluorescein construct demonstrated the ability to detect the target mRNA, showing that the final product is stable in a cellular environment and hybridised. Future work could be to use alkyne tagged-DNA or alkyne-tagged PNA probes and label them with tetrazine probes, such as those recently reported by Weissleder[134,135] that "switch-on" fluorescence only when conjugated.

Chapter V

PNA–Peptide Library

5. PNA–Peptide Library

Peptide libraries were synthesised with a PNA-tag attached, to allow hybridisation onto a DNA microarray. The peptide sequences chosen were designed to allow the interrogation of tyrosine kinase activity found within human leukemic tissue, with phosphorylation by aberrant overexpressed tyrosine kinases. This was achieved by the parallel synthesis of the desired PNA and peptide sequences, with the library assembled by ligation of each of the fragments.

5.1. Synthesis of PNA-tags

For the synthesis of the PNA-tags coupling on a diverse set of resins along with a variety of coupling methods were evaluated. The resins (characterised in **Table 5.1**) were used in combination with Oxyma/DIC or HBTU/DIPEA, with the synthesis of the PNA started by coupling of the Rink-linker. This was followed by coupling of the PNA monomers to form the tag sequence.

Table 5.1: Resins used for synthesis evaluation of PNA-tag's.

Name	*Abbreviation*	*Loading (mmol/g)*	*Swelling in DMF mL/g*
ChemMatrix	*CM*	*1.0*	*8*
Low Loading Polystyrene	*LL-PS*	*0.5*	*6*
TentaGel	*TG*	*0.21*	*5*
Polyethylene Glycol Polyacrylamide	*PEGA*	*0.4*	*11*

All of the couplings were carried out in a 96-well filter plate, as this was to be used to synthesise the full 100 PNA-tags. The coupling was left at 60 ˚C for 45 min, then cleaved using standard TFA conditions, and analysed by MALDI-TOF MS and HPLC. For the PNA synthesis Oxyma/DIC gave better results than the HBTU, and was therefore used for the full synthesis. As for the resin, LL-PS and CM showed

similar results, but due to the relative higher cost of CM, LL-PS was used for the synthesis of the library.

Satisfied with the optimisation of the coupling conditions for the well plate synthesis of the PNA, a liquid handling robot was programmed to disperse the desired activated monomer solution into the correct wells. All of the PNA sequences were designed to have melting temperatures between 34–36 ˚C (**Appendix 1**), and because of this, and for ease of synthesis, guanine was not used.

Once the programming was verified, PNAs synthesis was attempted. Ten random members of the library were selected (**Figure 5.1**), and after synthesis a small amount was of resin was cleaved and the PNA analysed, showing good crude purity by HPLC (65–78%, detection 254 nm). The remaining PNAs were synthesised in batches of 20 per plate. This also included the resynthesis of 5 PNA's which had a lower purity compared to the rest of the sequences.

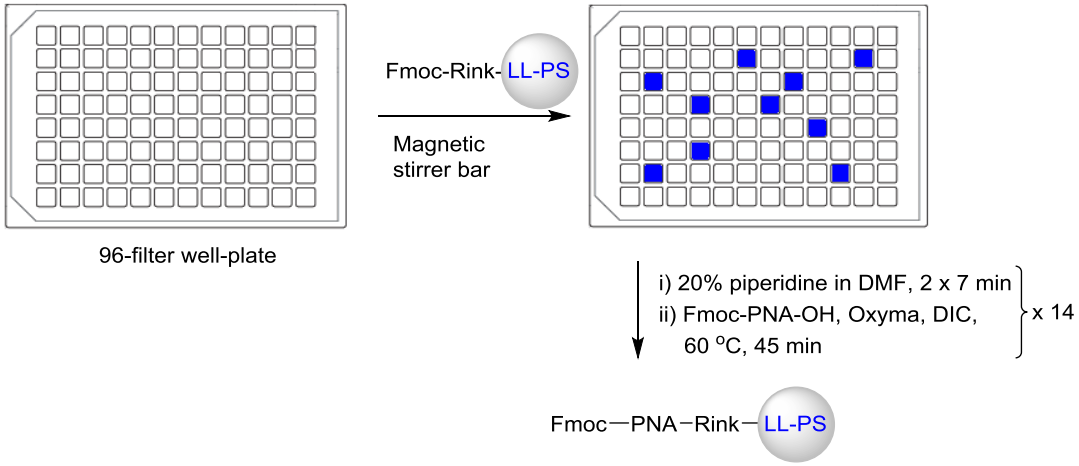


Figure 5.1: Parallel synthesis of PNA-tags using a 96-filter well plate with 20–30 mg of resin in each well.

After all 100 PNAs (**Appendix 1**) were synthesised, the members were stored in individual solid phase extraction (SPE)-tubes at −18 °C as Fmoc-protected sequences. A small sample of each PNA was taken and analysed by MALDI-TOF MS to validate if all the sequences had been synthesised. During this, it was noted that samples displayed masses of 2–6 Da more than the calculated mass. This was believed to be a calibration error and led to the inclusion of an internal standard

(containing Insulin and oxidized bovine Insulin B chain). One of the examples (**PNA049**) can be seen in **Figure 5.2**, showing both before (**top**) and after (**bottom**) calibration, giving the expected mass. Therefore all PNA's gave the expected mass following MS calibration.

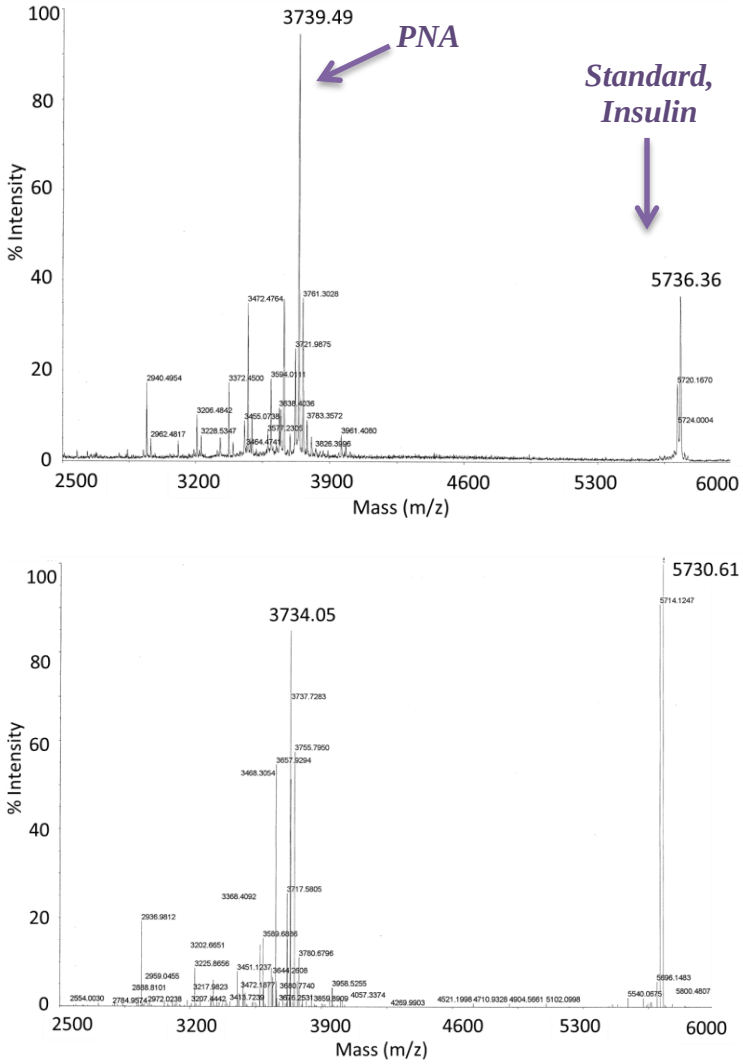


Figure 5.2: Analysis of **PNA049** (3733.49 Da) with an Internal standard (MALDI-TOF MS calibration: INS_BOVIN 3494.65 Da and 5730.61 Da); **Top:** MALDI-TOF-MS spectra of **PNA049** showing a mass of 3739.49 Da without calibration; **Bottom:** MALDI-TOF-MS spectra of **PNA049** showing a mass of 3734.05 Da with calibration.

After all PNAs were synthesised, Fmoc-Ahx-OH and 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (TZ-OH, **3.31**) were sequentially coupled, and the modified PNA's cleaved and purified by prep-HPLC. The yields after purification of the TZ-PNAs **5.1** ranged from 4–44%, and the PNAs were stored at −80 ˚C (ELSD-

HPLC and MALDI-TOF MS traces of pure **PNA028** and **PNA050** are shown in **Figure 5.3**). Lower yields can be attributed to loss of material during HPLC purification. None of **PNA035** (**Appendix 1**) was recovered.

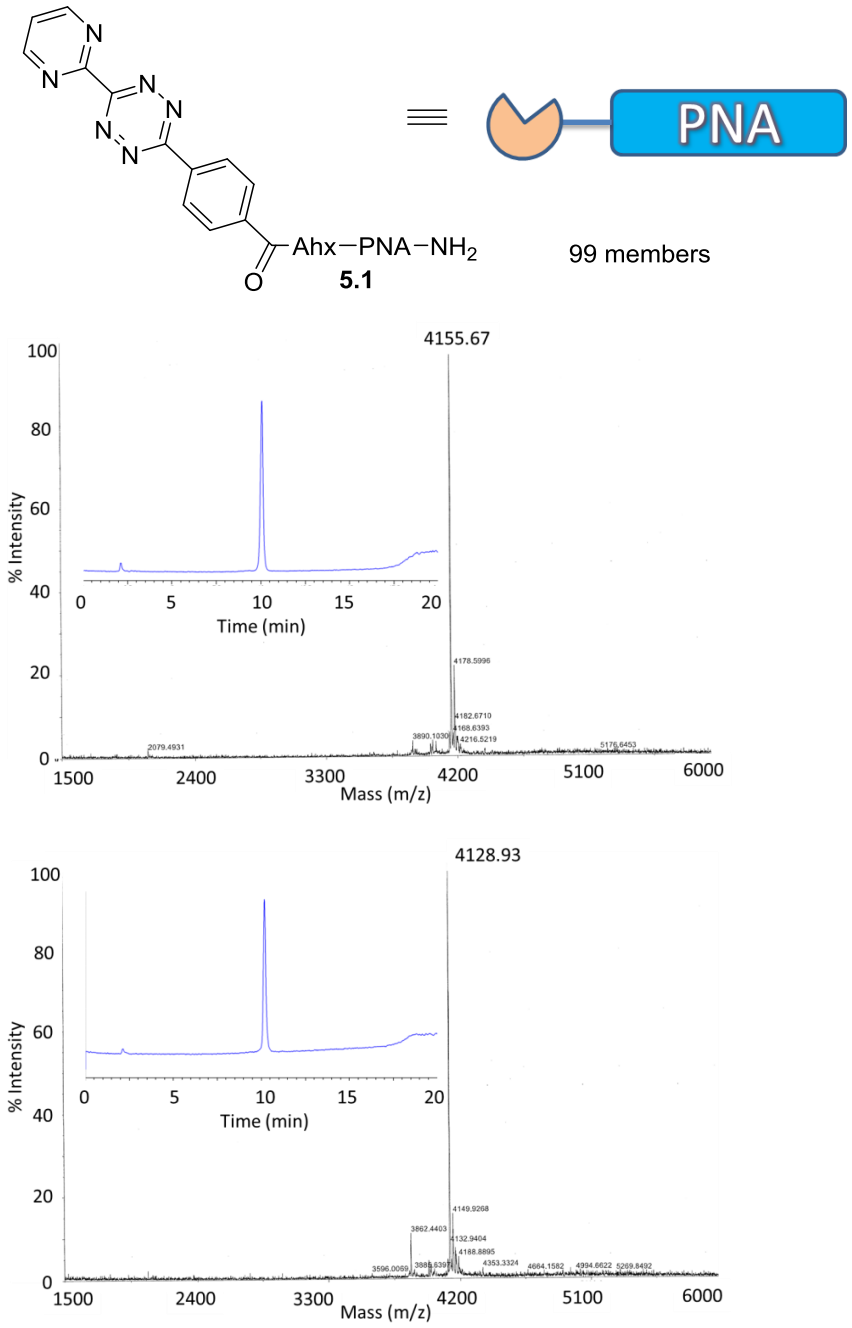
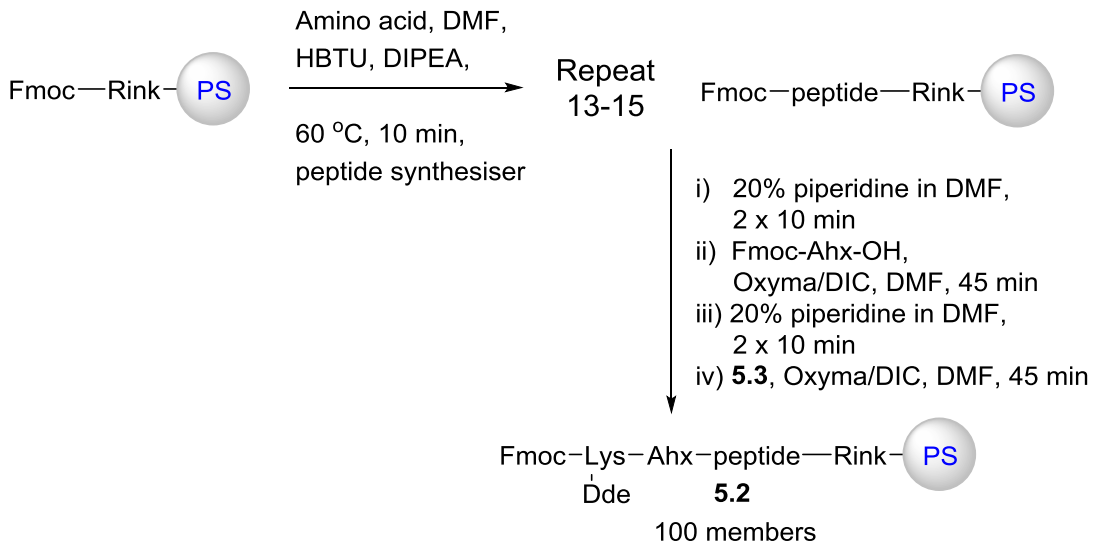


Figure 5.3: Structure of TZ-PNAs **5.1**; **Top:** chemical structure and generic representation of PNA's. HPLC with ELS detection (insert) and MALDI-TOF MS (without internal standard): **Middle: PNA028** (theoretical mass of 4144.69 Da); and **Bottom: PNA050** (theoretical mass of 4117.65 Da). (Note no internal calibration required subtraction of 11 Da to the observed masses).

5.2. Synthesis of peptide fragments

The corresponding peptide sequences synthesised (**Appendix 2**) were all known in literature to be phosphorylated solely by tyrosine kinases that are overexpressed in acute lymphoblastic leukaemia (ALL). Most sequences had seven amino acids on either side of the tyrosine, and did not contain cysteine or methionine. The absence of those two amino acids was intended to facilitate handling and synthesis of the library, eliminating the risk of oxidation.

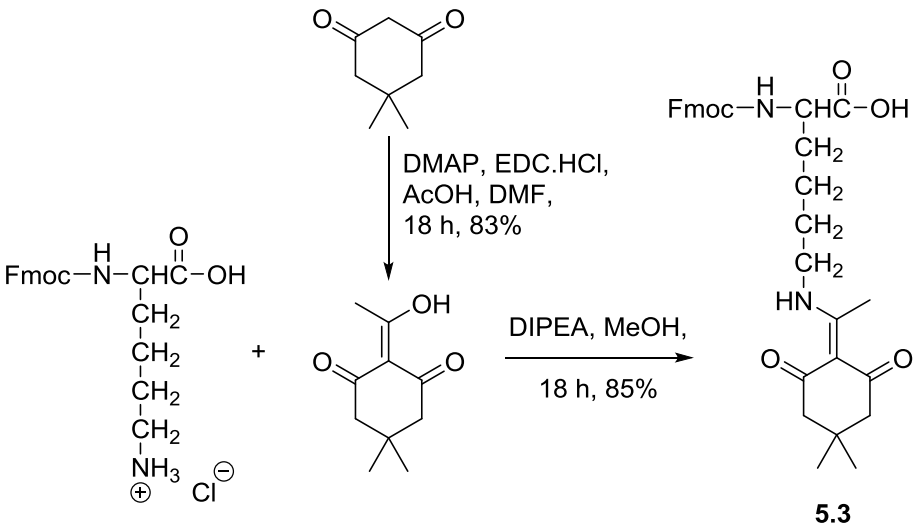
The peptides were synthesised on aminomethyl PS resin functionalised with a Rink-linker (0.75 mmol/g). The synthesis was carried out on a fully automated peptide synthesiser, which could synthesise two complete peptides during a 24 h period. After each peptide was completed, a small amount was cleaved and analysed, which resulted in the manual resynthesis of 21 peptides.



Scheme 5.1: Peptides were synthesised on Fmoc-Rink functionalised polystyrene resin (45 µmol), and the resin placed in a 12-channel CEM peptide synthesiser with microwave heating. After automated coupling of the amino acid sequence, Fmoc-Ahx-OH and Fmoc-Lys(Dde)-OH (**5.3**) were coupled manually and the resin stored at −20 °C.

For the automated synthesis HBTU/DIPEA was used in the couplings, because Oxyma decomposed in DMF during the long synthesis (12 h per run). For the manual synthesis, Oxyma/DIC was used. To all the peptides, aminohexanoic acid and Fmoc-Lys(Dde)-OH (**5.3**) were manually coupled using Oxyma/DIC.

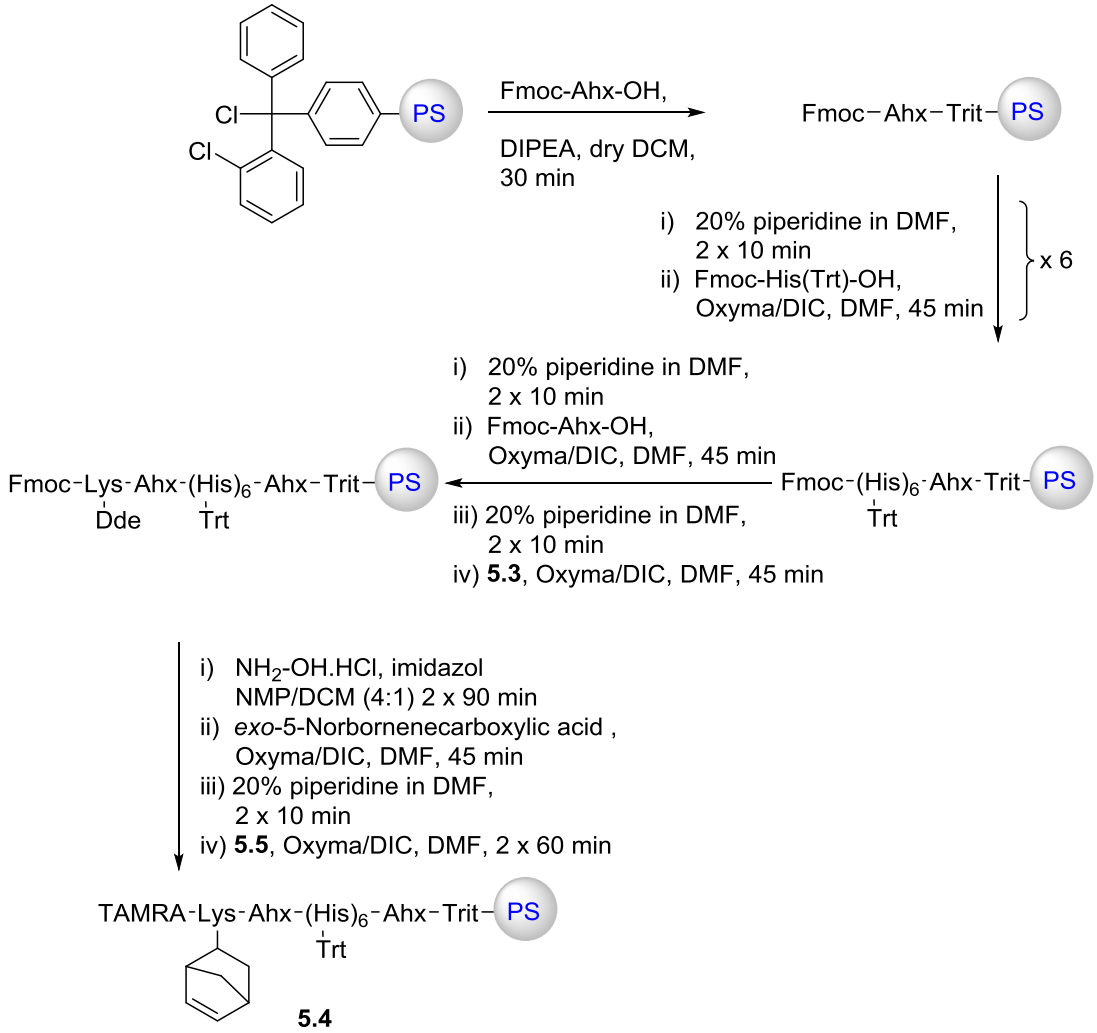
The problems with the hybridisation of the 10,000 membered library demonstrated the importance of a His-tag (**Chapter 2**) mediated purification. Thus TAMRA-Lys(Nor)-$(His(Trt))_6$-Ahx-OH (**5.4**, **Scheme 5.3**) was included in the library. His-tag **5.4** was synthesised on 2-chlorotrityl polystyrene resin, which was first activated by $SOCl_2$ in dry DCM, followed by the coupling of Fmoc-Ahx-OH in dry DCM/DIPEA. Six Fmoc-His(Trt)-OH residues were coupled followed by Fmoc-Ahx-OH and Fmoc-Lys(Dde)-OH (**5.3**, synthesised by coupling between the hydrochloric salt of Fmoc-Lys-OH and Dde-OH (**Scheme 5.2**)).[136,137]



Scheme 5.2: Synthesis of Fmoc-Lys(Dde)-OH **5.3**. Dde-OH was prepared by reacting acetic acid with 5,5-dimethylcyclohexane-1,3-dione in the presence of DMAP and EDC•HCl. Dde-OH was then coupled with Fmoc-Lys-OH•HCl in the presence of DIPEA.[136,137]

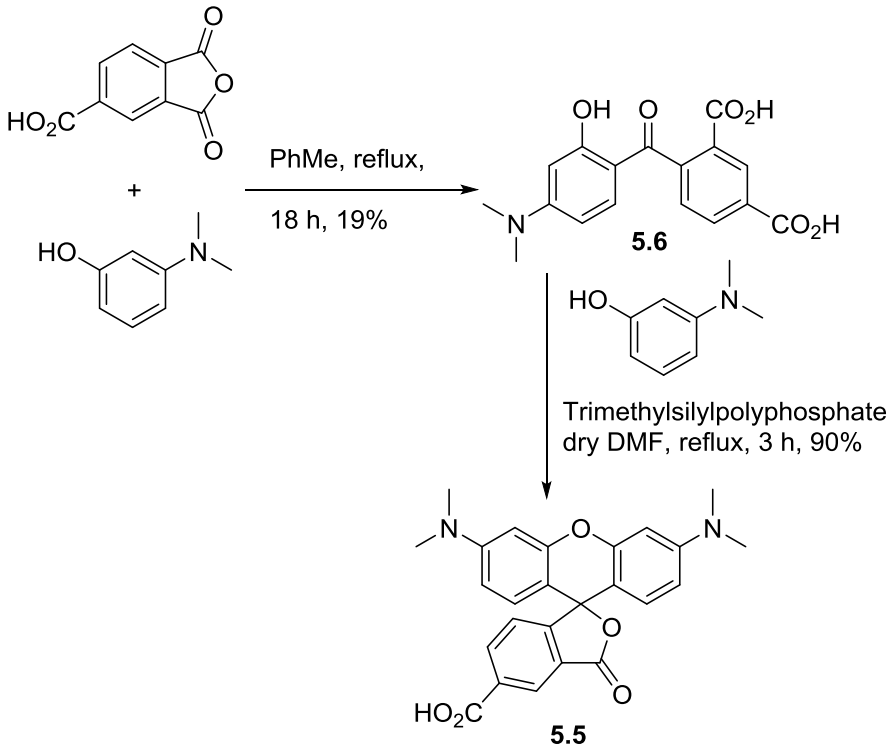
The Dde protecting group was cleaved using hydroxylamine hydrochloride and imidazole as described by Bradley *et al*.[29,41] *exo*-5-Norbornenecarboxylic acid

was coupled onto the free amino lysine side chain, followed by the coupling of 5-carboxytetramethylrhodamine (TAMRA, **5.5**) after Fmoc deprotection.



Scheme 5.3: TAMRA-Nor-His-tag **5.4** was synthesised on 2-chloro trityl functionalised polystyrene resin, by adding Fmoc-Ahx-OH, followed by Fmoc deprotection using piperidine. The coupling of Fmoc-His(Trt)-OH (repeated 6 times), was followed by the coupling of Fmoc-Ahx-OH and Fmoc-Lys(Dde)-OH (**5.3**). Finally, Dde was removed using NH_2-OH and the free amine coupled to *exo*-5-norbornenecarboxylic acid. The Fmoc-group was deprotected and TAMRA-OH (**5.5**) was coupled.

It is important to note that for this His-tag **5.4**, TAMRA **5.5** was used as a single isomer. This was because it would produce additional isomers during the ligation if more than one isomer of TAMRA was used. To synthesise the single TAMRA isomer, 3-dimethylaminophenol was first reacted with trimellitic anhydride in refluxing toluene. The product 4-dimethylamino-2-hydroxy-2′,4′-dicarboxy-benzophenone **5.6** was isolated by crystallisation form methanol as a red solid in a 19% yield. **5.6** was reacted with 3-dimethylaminophenol and trimethylsilyl-polyphosphate in dry DMF under reflux, to form the desired single isomer of TAMRA in 90% yield after purification (**Scheme 5.4**).[138]



Scheme 5.4: Synthesis of single isomer 5-carboxytetramethylrhodamine **5.5**. 3-dimethylaminophenol and trimellitic anhydride were refluxed in toluene. The 4-dimethylamino-2-hydroxy-2′,4′-dicarboxy-benzophenone **5.6** was crystallised from methanol, and refluxed with 3-dimethylaminophenol and trimethylsilylpolyphosphate in DMF for 3 h to give 4-TAMRA-OH (**5.5**) as a single isomer.

After the successful coupling of TAMRA, the protected His-tag **5.4** was cleaved using the optimised method (10% HFIP in $CHCl_3$). It proved very difficult to couple the His-Tag **5.4** to the 100 peptides. To test the coupling of His-tag **5.4** to the resin bound peptides **5.2**, five of the peptides were taken, and the protected His-tag was coupled using Oxyma and DIC. After 18 h, washing, and repeat of the coupling step, a Kaiser test[139] was carried out to verify the successful coupling. Thereafter the peptides were cleaved and analysed. The crude MALDI-TOF MS spectra indicated the presence of the desired product. Purification proved rather difficult, as for the first peptide purified none of the fractions collected showed the right mass in the MALDI-TOF MS spectra. Other peptides purified showed a mass corresponding to the unprotected His-tag and the mass of the product in the same fraction. Because the crude mass spectra of the first 5 peptides showed the desired peak, and while purifying these peptides, further 20 peptides were used to couple the His-tag. However, this proved to be more difficult as the Kaiser test remained positive after 3 couplings, and all of the His-tag was used up at this point. For these 20 peptides, most of them did not show the product peak in the crude MALDI-TOF MS, and purification of 14 of those peptides by HPLC did not show the right mass in any of the fractions, or the fractions showed both the His-tag and the product in the same fraction. In summary, of the 25 peptides **5.7** that were used by coupling the His-tag **5.4** to the peptide, only eight products showed the right mass, of which five showed the presence of unprotected His-tag in the mass spectra. It was thought that because of the resin used for the peptide synthesis (aminomethyl polystyrene with a loading of 1.23 mmol/g), the His-tag **5.4** (2987 Da) may have been to sterically hindered to couple onto the amine on the resin.

It was decided, therefore, to use 20 of the peptides and couple Rhodamine and norbornene directly without a His-tag (**Figure 5.4**). This peptide–PNA library would then be used for the hybridisation optimisation and enzyme tests. The peptide library **5.8** was synthesised by conjugating 5(6)-carboxyrhodamine (mixture of isomers was used as none of the single isomer of TAMRA was left and due to time constrains, no more could be synthesised) and *exo*-5-norbornenecarboxylic acid (as the single isomer) to 20 of the pre-synthesised resin bound peptides (**5.2**) using

Oxyma and DIC, followed by standard cleavage from the Rink-linker. All peptides were purified by HPLC and after freeze-drying the desired products obtained in 1–18% yield. The HPLC peptide purification method used for **5.8** separated the two isomers (due to the dye).

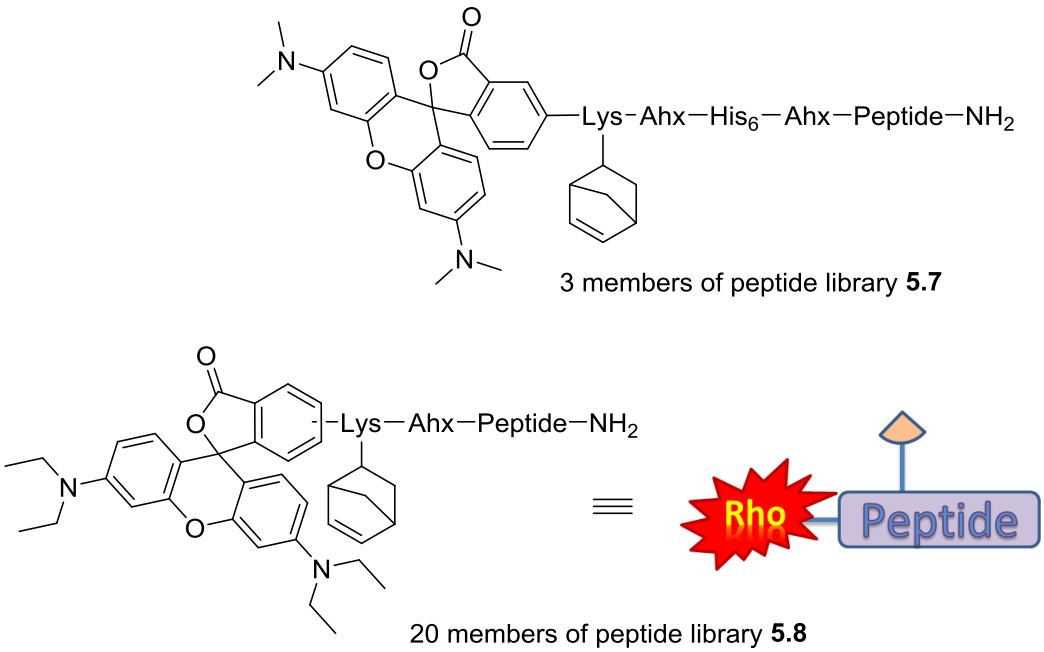
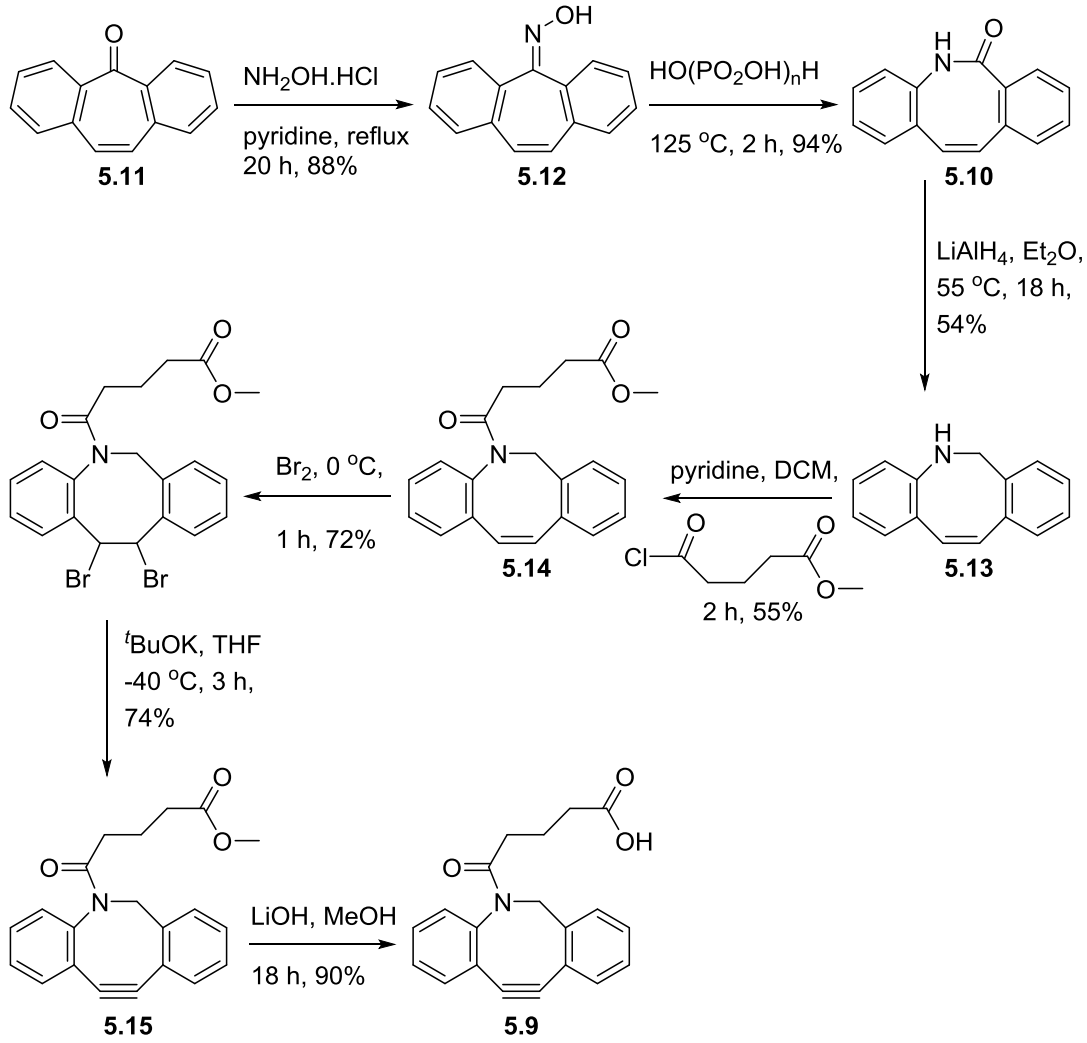


Figure 5.4: Top: First attempt of His-tag peptide library (**5.7**) synthesis, with His-tag coupled *via* peptide coupling, fluorophore: 5-TAMRA (**5.5**); and **Lower:** Peptide library **5.8** without His-tag, for enzyme test reactions, fluorophore: 5(6)-carboxy rhodamine.

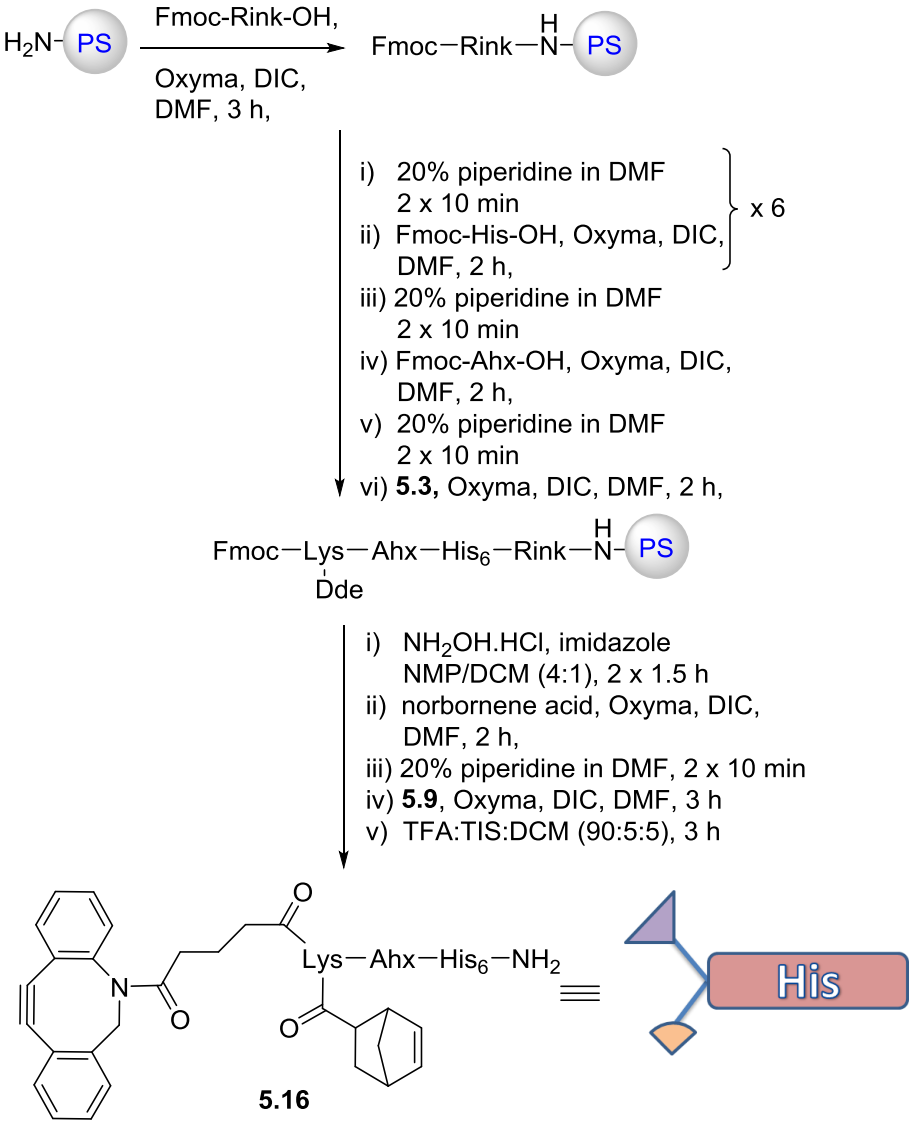
Thus, it was decided to try another method for solution phase ligation of the His-tag to the peptides. 5-(11,12-Didehydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-5-oxopentanoic acid **5.9** was synthesised (**Scheme 5.5**) in 7 steps from commercially available dibenzosuberenone with stepwise yields ranging from 54–89%. The Beckmann rearrangement was used to expand the seven membered ring to the eight membered to give **5.10**, by first reacting the aldehyde **5.11** with hydroxylamine hydrochloride to form the oxime **5.12** *via* a Schiff-base reaction. The oxime was heated in acid to initiate the rearrangement. The amide **5.10** was reduced to the amine **5.13** with $LiAlH_4$, followed by coupling of glutaric acid monomethyl ester chloride to form amide **5.14**. To introduce the alkyne functionality, the alkene was

first dibrominated using Br_2, followed by a di-dehydrobromination with ^tBuOK to form the alkyne ester **5.15**. Finally the acid **5.9** was obtained by ester hydrolysis of **5.15** with LiOH.



Scheme 5.5: Synthesis of 5-(11,12-didehydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-5-oxopentanoic acid **5.9** in 7 steps from dibenzosuberenone **5.11**.[105]

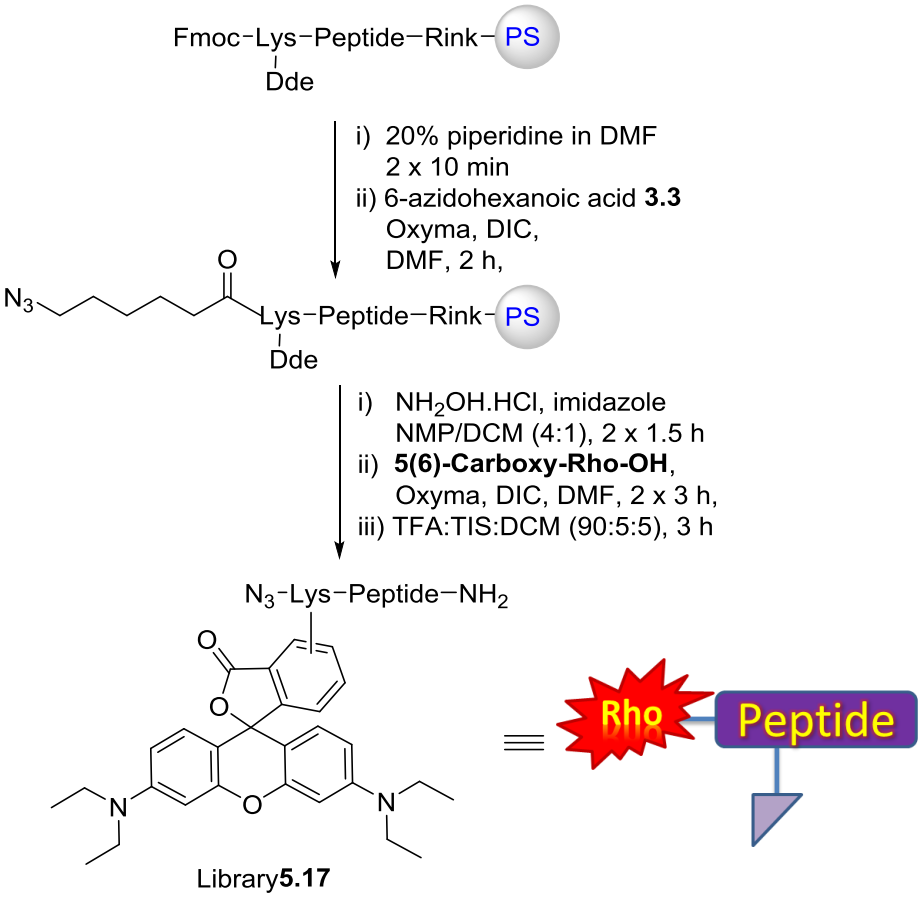
With the cyclic alkyne **5.9** in hand, the His-tag was resynthesised on Rink functionalised aminomethyl polystyrene resin (0.7 mmol/g, **Scheme 5.6**).



Scheme 5.6: Synthesis of a His-tag peptide with a norbornene group for tetrazine ligation to the PNA and a strained alkyne **5.9** for copper-free click ligation to the peptide.

Aminohexanoic acid was coupled as a spacer, followed by Fmoc-Lys(Dde)-OH **5.3**. After Dde-deprotection, norbornene was coupled to the resin and alkyne **5.9** was coupled after Fmoc-deprotection. The peptide was then cleaved from the Rink-linker using the TFA:TIS:DCM (90:5:5), and purified by HPLC to give the Alkyne-Nor-His-Tag **5.16**.

The second peptide library **5.17**, which will be used to couple the His-tag **5.16** *via* the copper-free click reaction to the peptide, followed by the ligation of the TZ-PNA to this conjugant. For this synthesis 37 of the resin bound peptides (**5.2**) were used to couple the readily available 5(6)-carboxyrhodamine, and the in-house prepared 6-azidohezanoic acid **3.3** (**Scheme 5.7**).

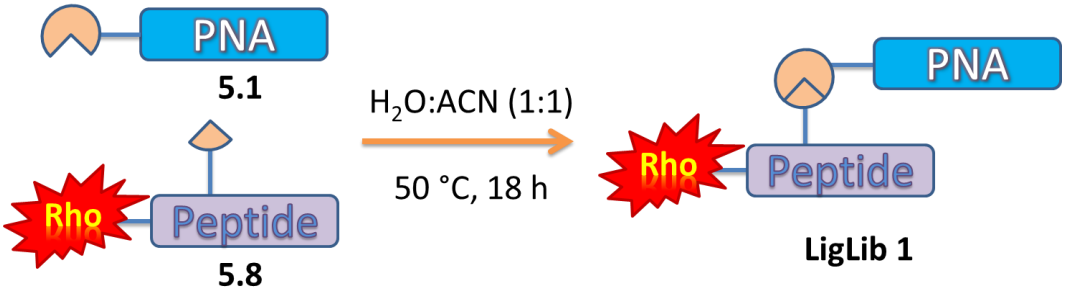


Scheme 5.7: Synthesis of peptide library **5.17** from resin bound peptides **5.2**. 37 peptides of **5.2** was coupled 6-azidohexanoic acid **3.3** after Fmoc-deprotection, followed by removal of Dde-OH using NH_2-OH and imidazole. 5(6)-Carboxy-Rho-OH was coupled and final deprotection and cleavage from the Rink-linker using TFA:TIS:DCM.

After the successful coupling with Oxyma and DIC of both the azide and Rhodamine, the peptides were cleaved using standard conditions and purified by HPLC, 34 members of the peptide library **5.17** were obtained in a yield of 1–19%.

5.3. Ligation of Peptides to PNA-Tags

To test the ligation of the peptide to the PNA, one peptide (**Pep001** (**Appendix 2**)) was ligated to **PNA001** (**Appendix 1**) using 1.5 eq of peptide (**Scheme 5.8**) to try and push the ligation to completion before decomposition of the TZ. The ligation was carried out in H_2O:ACN (1:1) containing 0.1% TFA.



Scheme 5.8: Ligation of 20 TZ-PNAs **5.1** and 20 members of the peptide library **5.8**.

After 8 h reaction, the ligation mixture was analysed by HPLC with ELSD detection, which showed the presence of both starting materials and an additional product peak. As the reaction was not completed, it was left for another 10 h, however, this did not make a major difference in the HPLC trace (HPLC after 18 h **Figure 5.5**). Once this test reaction and purification was carried out and the product collected, the remaining 19 ligations were carried out. The conjugates were recovered after HPLC purification and lyophilisation in a 16–76% yield with respect to the PNA used. **Lig010** showed no ligation.

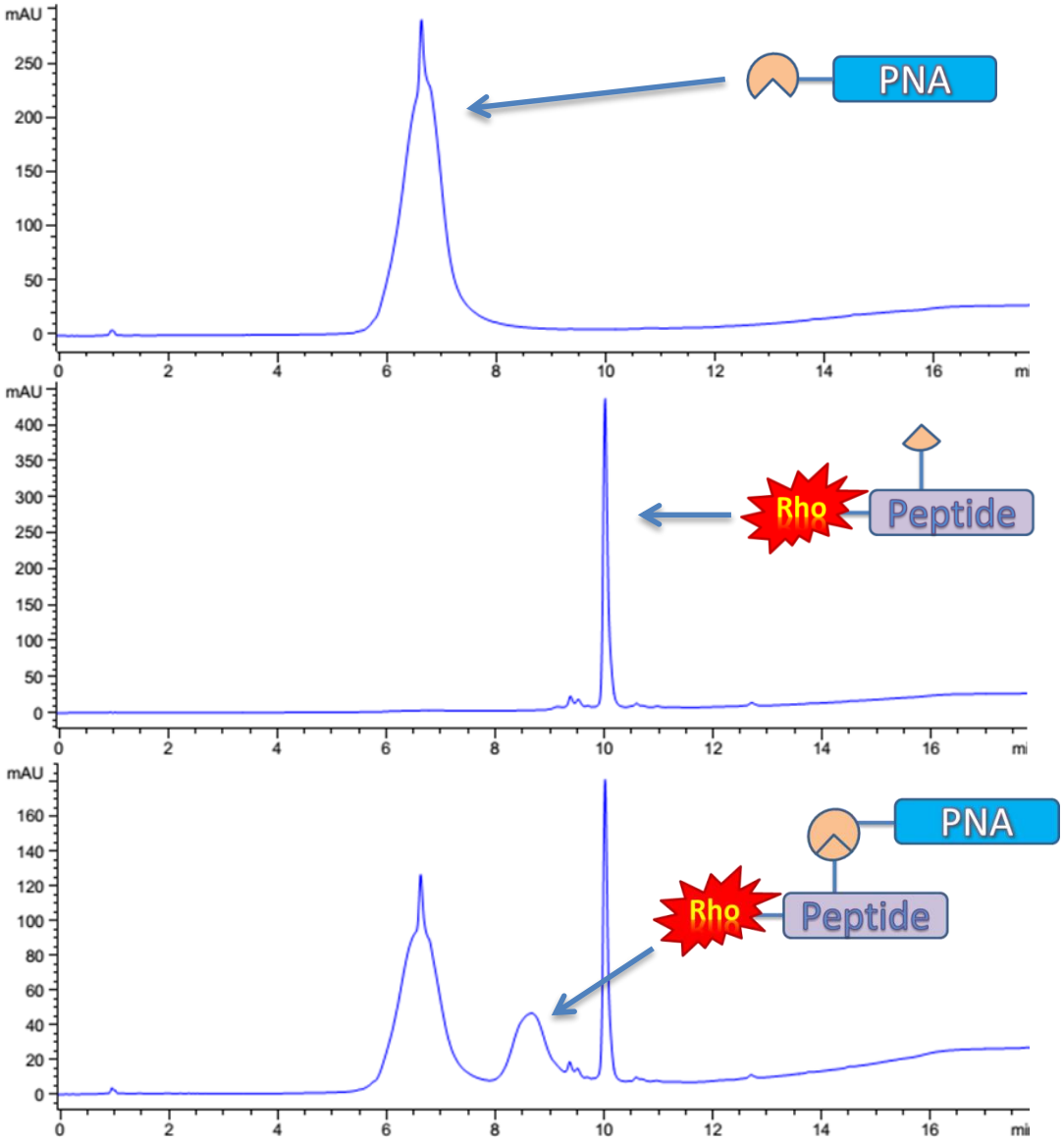
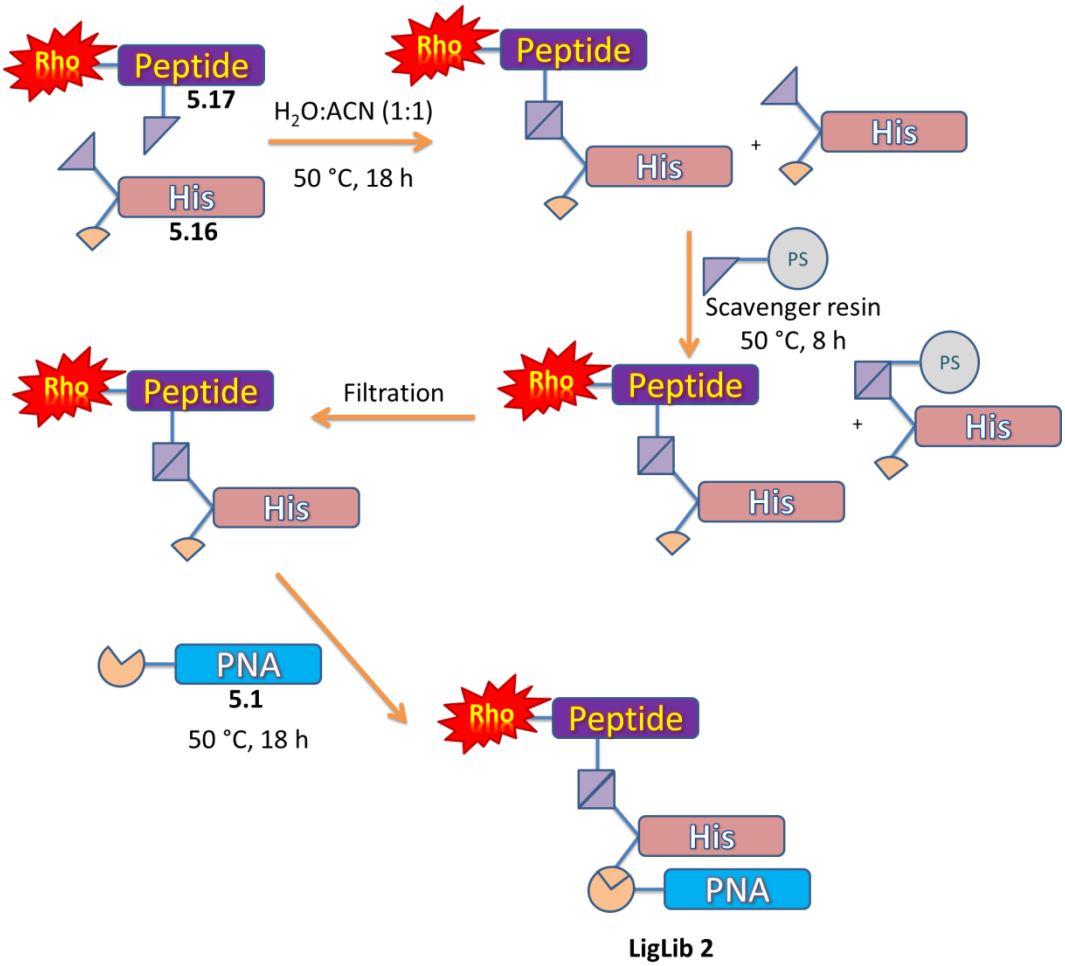


Figure 5.5: Top: HPLC spectrum at 254 nm of starting material **PNA100; Middle:** HPLC spectrum at 254 nm of **Pep002; Bottom:** HPLC spectrum at 254 nm of crude **Lig002**, showing product peak after 18 h reaction. The broad peaks of PNA were due to formic acid used for the analysis and not TFA.

As tetrazines react faster with cyclic alkynes than with norborene,[83] the order of addition for the ligation was important. First, the His-tag **5.16** was ligated using the cyclic alkyne to react with the azide of the peptide (strain-promoted alkyne–azide cycloaddition, or copper-free click), followed by the scavenging of any unreacted

alkyne using azide functionalised scavenger resin, and finally the ligation of the TZ-PNA **5.1** (**Scheme 5.9**). For **LigLib 2**, the first ligation between the 34 members of peptide library **5.17** and the His-tag **5.16** was left to react for 18 h in a 1:1 mixture of H_2O and acetonitrile (ACN) at 50 °C, followed by scavenging of any unreacted **5.16** by azide-resin scavenger pre-swollen in DCM.



Scheme 5.9: Ligation of His-tag **5.16** to the 34 members of peptide library **5.17**, followed by scavenger resin to remove unreacted **5.16**, and final ligation of 34 TZ-PNAs **5.1** to give 19 members of **LigLib 2** after purification.

This scavenger resin was prepared in house by coupling 6-azidohexanoic acid directly onto amino-methyl polystyrene resin. The scavenger resin, which reacted

with the excess of His-tag **5.16**, was filtered off and the filtrate was added to TZ-PNAs **5.1**. The tetrazine norbornene ligation was left to react for 18 h at 50 °C (**Scheme 5.9**). After HPLC purification, 19 out of the 34 members of this double ligated PNA-peptide library (**LigLib 2**) were obtained in 4–20% yields. The loss of members may be due to the fact that **5.16** hydrolysed over time, preventing the copper-free click reaction from proceeding. When the last crude conjugates were purified (detection/collection at 300 nm on the HPLC), it was noted that multiple additional peaks had appeared compared with the conjugates at the beginning. Those additional peaks, however, where not seen in at 530 nm detection wavelength. This would indicate that those extra peaks did not have Rhodamine coupled, and therefore the copper-free click reaction between the His-tag **5.16** and the peptide library **5.17** did not occur. When the His-tag **5.17** was re-analysed by MALDI-TOF MS, the mass of the peptide increased by 18 Da, indication that potential of the alkyne being hydrated over time. With the alkyne hydrated, it cannot undergo the ligation with the peptide, and it also cannot be scavenged by the resin, leaving the His-tag in the reaction mixture when it was added to the TZ-PNA **5.1**. This would allow the ligation between the tetrazine and norbornene of both, the hydrolysed His-tag and the peptide-His-tag fragment, to occur. This would account for the presence of the extra peaks, and the loss and lower yields of some of the members of **LigLib 2**.

In addition, it proved a great challenge to detect the desired mass (~8,000 Da) of each of the members in this **LigLib 2**. The detection difficulties may be due to the structure of the product, or the possibility of eight different isomers per member (**Figure 5.6**). The eight possible isomers arise from the orientation of the two ligation moieties (tetrazine and norbornene for the Diels-Alder reaction and the cyclic alkyne and azide for the copper-free click ligation) when they come together for the reaction to accrue. This gives rise to four different orientations in the product. The other isomers come from the Rhodamine used, as this was not a single isomer. HPLC purification of the peptide library **5.17** did not separate the isomers of all the peptides. Therefore some members of **LigLib 2** will have all eight isomers. Two isomers from the copper-free click reaction, another two from the Diels-Alder

ligation, giving four isomers for isomer of the peptide used (**Figure 5.6**). Therefore eight isomers if there were two isomers of rhodamine present in the peptide.

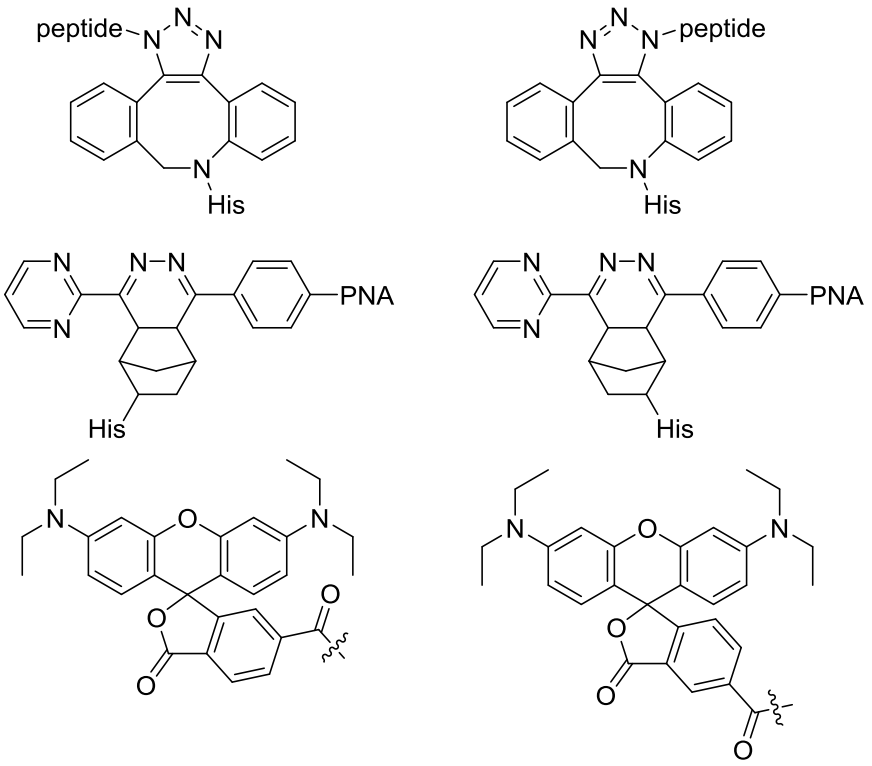


Figure 5.6: Possible isomers produced during the ligation of **LigLib 2**, and combinations of those account for the eight possible isomers in the members of **LigLib 2; Top:** Two isomers from the copper-free click reaction**; Centre:** Two isomers of the Diels-Alder reaction**;** and **Bottom:** Two isomers of Rhodamine used during the peptide synthesis.

In summary, after screening of resin and reaction conditions for the PNA synthesis, 100 PNA-tags were synthesised on solid phase in a 96 well-plate using Oxyma/DIC as coupling reagents. After the successful synthesis of the PNA sequences, TZ **3.31** was coupled to the *N*-terminus and the PNA-tags were cleaved off the resin and purified by HPLC. Following the PNA-tag synthesis, the corresponding 100 peptides were synthesised on an automated peptide synthesiser. Next, a side-chain protected His_6-tag sequence was synthesised on a chlorotrityl-PS resin, however, the subsequent coupling of the protected His-tag to the resin bound

peptides was unsuccessful. Therefore, ADIBO was synthesised and coupled to a His-tag to allow incorporation to the peptides in solution phase. Finally, this solution-based approach was applied to construct a PNA-encoded peptide library **LigLib 2** (19 members successfully obtained). In addition, a library (**LigLib 1**, 19 members) for the optimisation of the microarray hybridisation protocol was synthesised by functionalising the peptides with norbornene and TAMRA, followed by ligation to the TZ-PNA-tags.

Chapter VI

Conclusion

6. Conclusion

A new 10,000 membered cell penetrating PNA-encoded peptide library for the interrogation of tyrosine kinases was designed and successfully obtained using the split-and-mix method. Microarray analysis of cell lysate from K562 cells, after interrogation with the library, showed signal interfering presence of cell debris on the surface of the array. To overcome this, different detergents were screened, but they all hindered the hybridisation of the library onto the array. Therefore, a His-tag was added to the library members and then used with a $Ni^{(II)}$-column to successfully purify the library prior to hybridisation.

For the synthesis of a focused PNA-encoded library for known tyrosine kinases in leukaemia cells, different ligation methods were screen and optimised for the conjugation of a peptide to a PNA-tag. The copper catalysed cycloaddition reaction was problematic in the presence of PNA. This was overcome by utilising a TBTU (**1.8**) ligand in the reaction. However, this approach was not further developed as copper has been shown to interact with biological processes and traces of copper were still observed after purification. Another method ligation method, the inverse-electron demand Diels–Alder reaction between a tetrazine and an alkene, was investigated. For this, different tetrazines were synthesised and coupled to a resin bound PNA fragment. The *trans*-cyclooctane (**3.28**) and commercially available norbornene were coupled onto a peptide fragment, which was subsequently cleaved off the resin. The ligation of the peptide (functionalised with **3.28** or norbornene) to the chloro-tetrazine (**3.17**) PNA-tag on resin showed that this reaction did not go to completion. As access to TZ **3.17** was very difficult, TZ **3.20** was used instead. Unfortunately under these reaction conditions, the Diels–Alder reaction did not occur when norbornene was used. Screening of optimised reaction conditions for the ligation, using TZ **3.26** with norbornene **3.25,** showed that the norbornene and the tetrazine pair were not suitable the PNA-peptide ligation. Therefore, a more reactive tetrazine was synthesised instead.

The tetrazine ultimately used for ligation of the library was TZ **3.31**. Different reaction conditions were screened for the coupling of the carboxy-

functionalised tetrazine onto a PNA on a polymer resin, with Oxyma/DIC in DMSO/DCM (2 × 1 h) being the optimal conditions. However, the ligation of the peptides to the tetrazine functionalised PNA on solid phase was not sufficient and a solution phase ligation was attempted. For this, different conditions to cleave the TZ-PNA off the Rink-linker from polystyrene based resin were screened, with TFA:water (95:5) showing the best results. However with PEG-based ChemMatrix resin, TFA:phenol (95:5) was used.

Before construction of the full PNA-encoded peptide library, a FISH experiment was carried out to test the ligation in a biological system using tetrazine functionalised PNA-probes. These probes were targeting two different mRNA sequences in fixed HeLa cells. After incubation with the probes, a norbornene functionalised fluorescein was added to the cells. Analysis of the cells by fluorescent microscopy showed that the PNA-probes had hybridised to the desired mRNAs (fluorescein-functionalised PNA controls), but the fluorescent signal for the TZ-probes was much lower than expected. This could have been due to the ligation requiring a longer reaction time on the cells or the TZ degrading. To test this, norbornene functionalised dye was ligated with the TZ-PNA before incubation with the cells, showing that the Diels–Alder reaction did occur as indicated by a higher fluorescence compared with the intracellular ligation.

Finally the library targeting kinases in leukaemia was assembled but due to problems encountered with the split-and-mix library a His-tag was included. The His-Tag was first synthesised on a chlorotrityl-PS resin to allow peptide cleavage with side chain protecting groups intact, with a carboxy functionality at the *C*-terminus. The His-tag was coupled onto the *N*-terminus of the peptide sequences using an activated ester; however, after initial success the coupling was unsuccessful. As this was thought to be due to steric hindrance of the resin bound peptide, a different route (spAAC) was taken to incorporate the His-tag. A new His-Tag was synthesised on a Rink-linker, incorporating both ADIBO (**5.9**) and norbornene moieties. The peptides for the library were functionalised with TAMRA and an azide, whereas the PNA-tags were functionalised with the TZ. After purification of

all fragments, the library was synthesised by first reacting the azide-peptide with ADIBO-His-tag (through the spAAC reaction), followed by the scavenging of any unreacted ADIBO-His-tag using azide-PS resin. Finally, the TZ-PNA-tag was ligated with the norbornene of His-Tag–peptide fragment. Due to hydration of the alkyne, and loss of members through the purification method, only 19 members of this library were obtained.

As the His-tag addition to the library was problematic, a second library was synthesised, which could be used for optimisation or reaction conditions for the microarray analysis and enzyme screening test. For this the peptides were functionalised with TAMRA and norbornene and the PNA-tags with the tetrazine. After the purification of each member and tag the ligation between the peptide and PNA accrued at 50 °C for 18 h. However, HPLC analysis showed, that purification was needed. After purification, 19 members of this second library were obtained.

Chapter VII

Experimental

7. Experimental

7.1. General section

Reactions involving moisture sensitive reagents were performed under a positive pressure of nitrogen. Evaporation of solvents was performed at reduced pressure, using a Büchi rotary evaporator. All chemicals were purchased from Aldrich, Acros, LinkTec or Fluka and used as received. Microwave assisted heating was carried out by irradiating the reaction mixture in a Biotage® Initiator at 2.45 GHz.

^{1}H and ^{13}C **NMR** spectra were recorded on a Bruker AVA400 (400 and 100 MHz respectively) or AVA500 spectrometer (500 and 126 MHz respectively) at 298 K in deuterated solvents. Residual protic solvent $CHCl_3$ (δ_H = 7.26 ppm), methanol (δ_H = 3.30 ppm) and DMSO (δ_H = 2.50 ppm) or deuterated solvent $CDCl_3$ (δ_C = 77.0 ppm), D_4-methanol (δ_C = 49.0 ppm) and D_6-DMSO (δ_C = 39.5 ppm) were used as an internal reference. Peak positions are reported as downfield shifts in parts per million (δ) from TMS. Coupling constants were measured in hertz (Hz). Resonances are characterised as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), multiplet (m), or broad singlet (bs).

Aluminium-backed silica plates (Merck silica gel 60 F_{254}) were used for **thin layer chromatography** (TLC) to monitor solution-phase reactions. TLC visualisation was carried out using ultraviolet (UV) light at 254 nm or by staining with of phosphomolybdic acid (PMA) solution. Flash column chromatography was performed on silica gel using Kieselgel 60, 230-400 mesh (Merck).

MALDI-TOF MS analysis was performed on an Applied Biosystems Voyager DESTR MALDI-TOF mass spectrometer and a Bruker ultrafleXtreme MALDI-TOF MS. Sinapinic acid was used as a matrix and positive or negative ion mass spectra reported. **Chemical or electrospray ionisation mass spectrometry** (CI/ESI-MS) analyses were performed on an Agilent Technologies LC/MSD Series 1100/1200 quadrupole mass spectrometer. Product peaks are reported with percentage intensities of the base peak.

HPLC was carried out on an Agilent Technologies 1100/1200 Chemstations coupled to a Polymer Lab 100 ES evaporative light scattering detector (ELSD). Solvents used were (A) H_2O/0.1% formic acid, (B) CH_3CN/0.1% formic acid, (C) MeOH/0.1% formic acid, (D) H_2O/0.1% TFA and (E) CH_3CN/0.1% TFA, at a flow rate of 1 mL/min. The columns used were a Discovery C18 from Supelco (50 mm × 4.6 mm, 5 µm) or Poroshell 120 SB-C18 from Agilent (50 × 4.6 mm, 2.7 µm) for methods 1+2, and an Acclaim® 120 C18 from Dionex (150 × 4.6 mm, 5 µm) for method 3+4. The following methods were used:

- *Method 1* [eluents (A) and (B)]: 95% to 5% (A) over 10 min, 5% (A) for 4 min.

- *Method 2* [eluents (A) and (C)]: 95% to 5% (A) over 10 min, 5% (A) for 4 min.

- *Method 3* [eluents (D) and (E)]: 95% (E) for 2 min, 95% to 50% (E) over 8 min, 50% to 5% (E) over 4 min, 5% (E) for 2 min.

- *Method 4* [eluents (D) and (E)]: 95% (E) for 2 min, 95% to 50% (E) over 13 min, 50% to 5% (E) over 5 min, 5% (E) for 4 min.

Preparative HPLC: ZORBAX 300SB-C18 preparative column (250 × 9.4 mm, 5 μm, 300 nm pore size), at a flow rate of 2 mL/min with eluent D and E were used for purification. The following gradients were used **for PNA** purification: 90% (D) for 3 min, 90% (D) to 50% (D) over 22 min, 50% to 5% (D) over 5 min, 5% (D) for 5 min, collection by UV at 300 or 580 nm, **and peptide:** 95% (A or D) to 40% (A or D) over 40 min, 40% to 5% (A or D) over 5 min, 5% (A or D) for 5 min, detection by UV at 254 or 600 nm.

The **liquid handler** used was a 4-Tip MultiPROBE II EX systems from Packard, with customised programming of the WinPREP software. The **peptide synthesiser** was a CEM Liberty 12-Channel Automated Microwave Peptide Synthesiser with the PepDrive software, using *N,N,N′,N′*-tetramethyl-O-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) as the coupling agent. For single peptide and PNA synthesis a semi-automated peptide synthesiser, **Biotage**

Initiator+ SP Wave, was used with pre-activated monomers using Oxyma and *N*,*N*'-diisopropylcarbodiimide (DIC) in dimethylformamide (DMF).

7.2. General experimental procedures

7.2.1. Peptide coupling

Suitably protected Fmoc-amino acids (3 eq) and Oxyma or hydroxybenzotriazole (3 eq) were dissolved in DMF (0.1 M) and mixed for 10 min. To the resulting solution, DIC (3 eq) was added and the mixture was stirred for an additional 5 min. The activated acid was added to the amino functionalised resin (1 eq), pre-swollen in dichloromethane (DCM), and the reaction was mixed for 3 h at room temperature or 30 min at 60 °C. The resin was washed with DMF (× 3), DCM (× 3) and methanol (× 1). The coupling reaction was confirmed by a Ninhydrin test.[139]

7.2.2. Fmoc deprotection

Fmoc deprotection was carried out by pre-swelling the resin with DCM, followed by stirring/mixing with 20% piperidine in DMF for 2 × 7 min.

7.2.3. Dde deprotection in presence of the Fmoc group

A solution of hydroxylamine hydrochloride (1.3 eq) and imidazole (1 eq) in *N*-methyl-2-pyrrolidone (0.27 M) was diluted with DCM (for PS and TentaGel resin) or DMF (PEGA resin), respectively in a 4:1 ratio. This solution was added to the resin, mixed for 2 × 1.5 h, washed with DMF (× 3), DCM (× 3) and methanol (× 3).[41]

7.2.4. Dde deprotection in absence of the Fmoc group

A freshly prepared solution of 4% hydrazine in DMF was added to the resin and left shaking for 5 × 10 min, and washed with DMF (× 3), DCM (× 3) and methanol (× 3).[41]

7.2.5. Cleavage from the Rink-linker and deprotection

A cleavage cocktail was freshly prepared using either trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/DCM (90:5:5) or TFA/water (95:5 for tetrazine containing PNA and peptides on PS resin) or TFA/phenol (95:5 for tetrazine containing PNA and peptides on CM resin) or reagent K (TFA/TIS/DCM/phenol, 88.5:2.5:5:5, for Arg containing peptides). The cocktail solution (twice the volume of the swollen resin) was added to the pre-swollen resin and agitated for 3 h. The solution was collected by filtration and the peptide precipitated from the cleavage solution by the addition of cold Et_2O (1:15 v/v). The peptide was collected by centrifugation for 10 min (~7000 RPM), and the liquid was decanted. The peptide was re-dispersed in cold ether and centrifuged (× 3).

7.2.6. Cleavage from the 2-chloro trityl linker

The resin was swollen in DCM for 10 min, followed by filtration and addition of a solution of 1% TFA in DCM. After 2 min the solution was added to a solution of 10% pyridine in methanol. This step was repeated 6–10 times. The combined solutions were concentrated under reduced pressure, and the protected peptides were precipitated from cold ether.

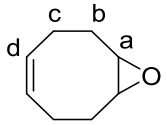
7.2.7. Microarray experiment

The PNA-peptide library (2 nM) was incubated with the recombinant kinase (Abl1, P3049, Invitrogen, 60 units) or cell lysate (from 3×10^6 cells), 2× kinase buffer (50 µL, solution contains: 100 mM Tris (pH 7.5), 20 mM $MgCl_2$, 4 mM $MnCl_2$, 2 mM dithiothreitol, 10 mM ATP) and protease and phosphatase inhibitor cocktails (for cell lysate only, 1 µL, Sigma Aldrich) as a solution (100 µL) between 1 h and shaken overnight at 37 °C. To this solution was then added GenHyb buffer (Genetix, 100 µL), and denatured at 90 °C for 10 min. The hybridisation solution (100 µL) was added using pre-heated (60 °C) pipet tips onto the pre-heated cover slide. The pre-heated microarray (custom made by OGT) was then placed on top of the cover slide and placed in the pre-heated hybridisation chamber. The hybridisation was done by lowering the temperature every 30 min in a hybridisation oven from 65 to 55 to 50 to 48 to 46 to 44 to 42 to 40 to 37 °C, followed by overnight at 37 °C.

For the detection, the microarray was washed for 10 min at 30 °C with wash buffer (pH 7.5, 2 × 45 mL, solution: 100 mM NaCl, 10 mM citric acid, 0.7% (w/v) *N*-lauroyl-sarcosine sodium salt, 0.1 mM ethylene glycol tetraacetic acid), followed by distilled water (45 mL,) and blocked with TPBS (45 mL, solution: 0.05% Tween-20 in PBS (phosphate buffered saline), pH 7.4) containing 1% BSA (bovine serum albumin) for 1 h at 30 °C. Then the array was washed with distilled water (45 mL), and labelled with an anti-phospho-tyrosine antibody (PY-20, P4110 from Sigma Aldrich, 100 µL, in a 1/500 dilution with TPBS/1% BSA) in the hybridisation chamber for 1 h at 30 °C. This was followed by washing with TPBS/1% BSA (2 × 45 mL) for 1 h at 30 °C and labelling with a secondary antibody (Cy5-goat anti-mouse IgG (H+L), 100 µL, in 1/200 dilution with TPBS/1% BSA) for 10 min at 30 °C. Finally, the array was washed with TPBS/1% BSA (2 × 45 mL) for 10 min at 30 °C, rinsed with distilled water (45 mL) and dried, before detection using a microarray scanner.

7.3. Chapter 3

7.3.1. (Z)-4-Cyclooct-1-ene monoepoxide (3.12)



To a solution of *cis*-1,5-cyclooctadiene (5.0 mL, 4.0 g, 40 mmol, 1 eq) in $CHCl_3$ (100 mL) at 0 ˚C was slowly added over 2 h a mixture of 3-chloroperoxybenzoic acid (9.58 g, 55.5 mmol, 1.36 eq) in $CHCl_3$ (100 mL). The reaction mixture was stirred at room temperature for 18 h, followed by removal of solid residues. The filtrate was washed with sat. $NaHSO_4$ (100 mL), sat. $NaHCO_3$ (100 mL) and sat. NaCl (100 mL). The organic layer was dried (anhyd. $MgSO_4$), filtered and evaporated under reduced pressure to give an oily crude product. (*Z*)-4-Cyclooct-1-ene monoepoxide **3.13** was isolated by flash column chromatography (eluting with 10% EtOAc/hexane) as a colourless oil (3.5 g, 69%).[104]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.64-5.58 (2H, m, C^d*H*=C*H*), 3.09-3.05 (2H, m, C^a*H*-O), 2.50-2.14 (4H, m, C^b*H*$_2$-CO), 2.10-2.03 (4H, m, C^c*H*$_2$-C=C)

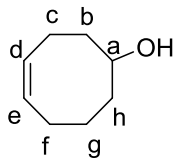
^{13}C NMR (125 MHz, $CDCl_3$): δ_C 128.9 (H*C*=), 56.7 (*C*HO), 28.1 (*C*H$_2$), 23.7 (*C*H$_2$).

HPLC (method 1): t_R = 11.6 min (88% purity, ELSD)

MS (CI) *m/z*: 124.1 [M^+, 23%]

R_f (10% EtOAc/hexane): 0.63

7.3.2. (Z)-4-Cycloocten-1-ol (3.13)



A suspension of $LiAlH_4$ (4.95 g, 130.4 mmol, 3 eq) was prepared in anhyd. THF (250 mL) and stirred at 0 °C in an ice-bath. A solution of cyclooctene monoepoxide **3.12** (5.54 g, 44.7 mmol, 1 eq) in dry THF (25 mL) was slowly added over 15 min. The reaction mixture was warmed to room temperature and refluxed for 4 h. The solution was cooled to 0 °C, and water (5.5 mL) was added drop wise, followed by addition of 15% *aq* NaOH (5.5 mL) and finally, water (40 mL). The resulting mixture was allowed to stir for 3 h, during which time the grey solid turned white. Celite and anhyd. $MgSO_4$ were added and stirred for 30 min, followed by filtration and washing with DCM (2 × 50 mL). The combined organic filtrates were dried (anhyd. $MgSO_4$), filtered and the solvent evaporated to yield the desired alcohol as a colourless oil (5.52 g, 98%), which was used in the next step without further purification.[104]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.72-5.67 (1H, m, C^e*H*=CH), 5.62-5.57 (1H, m, C^f*H*=CH), 3.82 (1H, m, C^a*H*-OH), 2.33-2.27 (1H, m), 2.14-2.08 (3H, m), 1.96-1.83 (2H, m), 1.74-1.61 (2H, m), 1.58-1.48 (2H, m).

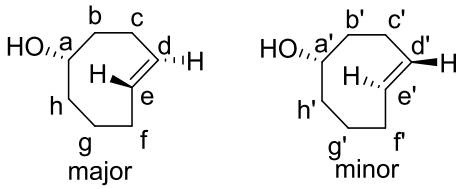
^{13}C NMR (125 MHz, $CDCl_3$): δ_C 130.2 (*C*H), 129.6 (*C*H), 72.8 (*C*HO), 37.8 (CH_2), 36.3 (CH_2), 25.7 (CH_2), 24.9 (CH_2), 22.8 (CH_2).

HPLC (method 1): t_R = 10.0 min (67% purity, ELSD)

MS (CI) *m/z*: 127.1 [$(M+H)^+$, 45%]

R_f (10% EtOAc/hexane): 0.2

7.3.3. (E)-4-Cycloocten-1-ol (3.27)



(*Z*)-4-Cycloocten-1-ol **3.13** (1.99 g, 15.7 mmol) was dissolved in a mixture of Et_2O/hexane (9:1, 50 mL), and methylbenzoate (2.5 mL, 17.4 mmol, 1.1 eq) was added. The reaction mixture was transferred to a quartz tube, placed into a light reactor, and irradiated at 254 nm. After 30 min of irradiation the tube was removed from the reactor and the reaction mixture was filtered through a column filled with 10% silver nitrate on silica (30 g). The starting material was eluted with Et_2O (100 mL) from the silver nitrate column, evaporated and re-dissolved in the reaction solvent. The solution was placed into the quartz tube and continued to be irradiated. This cycle was repeated 12 times. After this the column was washed with fresh solvent of Et_2O/hexane (9:1, 300 mL). The silica was placed in a beaker and 30% *aq* ammonia (150 mL) and DCM (150 mL) was added. After 15 min the solution was filtered, and the organic layer was separated. The aqueous layer was washed with DCM (2 × 50 mL) and the combined organic layers were washed with water (150 mL), dried with anhyd. $MgSO_4$, filtered and evaporated. The product was purified by column chromatography (silica, eluent: petroleum ether:EtOAc 9:1), to yield the two isomers as a pale yellow oil (major: 310 mg, 16%, minor: 227 mg, 12%). The isomers were identified by comparison to the literature and NMR analysis.[104,140]

Major isomer:

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.60-5.54 (1H, m, C^d*H*=CH), 5.42-5.35 (1H, m, C^e*H*=CH), 3.48-3.45 (1H, m, C^a*H*-OH), 2.36-2.25 (3H, m), 1.98-1.90 (4H, m), 1.71-1.58 (3H, m).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 135.1 (*C*H), 132.8 (*C*H), 77.8 (*C*HO), 44.6 (CH_2), 41.1 (CH_2), 34.3 (CH_2), 32.7 (CH_2), 31.2 (CH_2).

HPLC (method 1): t_R = 0.7 min (99% purity, ELSD)

MS (CI) *m/z:* 125.1 [$(M-H)^-$, 100%]

R_f (petroleum ether/ EtOAc 5:1): 0.2

Minor isomer:

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.58-5.57 (2H, m, $C^{d'}H$=$C^{e'}H$), 4.06-4.02 (1H, m, $C^{a'}H$-OH), 2.42-2.34 (1H, m), 2.27-2.20 (2H, m), 2.17-2.07 (2H, m), 1.90-1.75 (4H, m), 1.69-1.62 (1H, m).

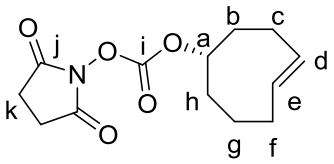
^{13}C NMR (100 MHz, $CDCl_3$): δ_C 134.3 (*C*H), 133.1 (*C*H), 67.4 (*C*HO), 43.0 (CH_2), 34.1 (CH_2), 34.0 (CH_2), 29.3 (CH_2), 27.7 (CH_2).

HPLC (method 1): t_R = 10.5 min (84% purity, ELSD)

MS (CI) *m/z:* 125.1 [$(M-H)^-$, 50%]

R_f (petroleum ether/EtOAc 5:1): 0.55

7.3.4. (E)-Cycloocten-4-yl 2,5-dioxopyrrolidin-1-yl carbonate (3.28)



(*E*)-4-Cycloocten-1-ol **3.27** (minor isomer, 200 mg, 1.8 mmol) and triethylamine (1 mL, 7.2 mmol, 4 eq) were dissolved in anhydrous acetonitrile (12 mL). To this solution *N*,*N*-disuccinimidyl carbonate (1.01 g, 3.9 mmol, 2.1 eq) was added slowly, and the reaction mixture was stirred for 72 h. The volatiles were evaporated and the remaining residue was taken up in ether, washed with 0.1 M HCl (20 mL), water (2 ×

20 mL) and dried with anhyd. $MgSO_4$. The ether was evaporated and the resulting oil was purified by column chromatography (1:1 ether:hexane) yielding a pale yellow viscous oil (150 mg, 31%) and recovered starting material (51 mg, 25%).[104]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 5.76-5.69 (1H, m, C^d*H*=CH), 5.65-5.59 (1H, m, C^e*H*=CH), 5.05-5.02 (1H, m, C^a*H*O), 2.90 (4H, s, 2 × C^k*H*$_2$), 2.55-5.38 (3H, m), 2.24-2.20 (1H, m), 1.97-1.90 (2H, m), 1.83-1.68 (2H, m), 1.49-1.31 (2H, m).

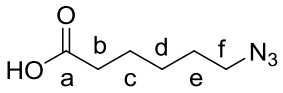
^{13}C NMR (125 MHz, $CDCl_3$): δ_C 168.8 (2 × q*C*), 151.0 (q*C*), 135.7 (*C*H), 131.2 (*C*H), 78.6 (*C*HO), 40.5 (*C*H$_2$), 34.1 (*C*H$_2$), 32.1 (*C*H$_2$), 29.6 (*C*H$_2$), 27.6 (*C*H$_2$), 25.5 (2 × *C*H$_2$).

HPLC (method 1): t_R = 8.2 min (85% purity, ELSD)

MS (ESI) *m/z:* 265.2 [(M-2)$^+$, 62%]

R_f (1:1 ether/hexane): 0.13

7.3.5. 6-Azidohexanoic acid (3.3)



To a stirred solution of 6-bromohexanoic acid (5.12 g, 26.2 mmol) in DMF (16 mL) was added NaN_3 (3.40 g, 52.3 mmol, 2 eq) and the mixture was heated at 85 °C for 3 h. The crude reaction mixture was diluted in DCM (350 mL) and washed with 0.1 N HCl (2 × 250 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated under reduced pressure to yield the product (3.75 g, 91%) as an oil, which was used in the next step without further purification.[141]

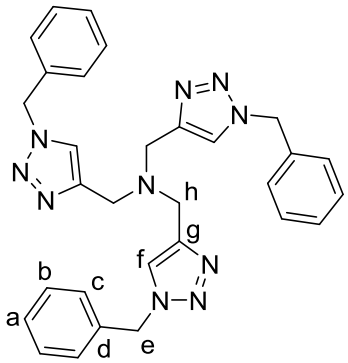
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 3.28 (2H, t, *J* = 6.9 Hz, C^b*H*$_2$C(O)O), 2.39 (2H, t, *J* = 7.4 Hz, C^f*H*$_2$N$_3$), 1.70-1.59 (4H, m, 2 × C^{c,e}*H*$_2$CH$_2$), 1.47-1.41 (2H, m, CH$_2$C^d*H*$_2$CH$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 178.5 (*qC*(O)O), 51.2 (*C*H_2CO_2H), 33.7 (*C*H_2), 28.6 (*C*H_2), 26.2 (*C*H_2), 24.2 (*C*H_2).

HPLC (method 1): t_R = 0.7 min (99% purity, ELSD)

MS (ESI) *m/z*: 156.1 [$(M-H)^-$, 100%

7.3.6. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 1.8)



Benzyl azide (333 mg, 2.5 mmol, 3.3 eq) and trispropargyl amine (100 mg, 0.76 mmol, 1.0 eq) were dissolved in DCM (0.7 mL), and water (0.7 mL) was added. $CuSO_4$•5 H_2O (10 mg, 0.04 mmol, 0.05 eq) and sodium ascorbate (23 mg, 0.12 mmol, 0.15 eq) were added and the reaction mixture was stirred vigorously overnight. DCM (7 mL) and water (7 mL) were added and the phases were separated. The aqueous phase was extracted with DCM (2 × 20 mL) and the combined organic phases dried (anhyd. $MgSO_4$), evaporated and purified by flash column chromatography (silica, DCM/methanol 95:5). The resulting residue was dissolved in a small volume of DCM, precipitated in hexane and filtered to give TBTA (251 mg, 63%) as a colourless, amorphous solid.[142]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.65 (1H, s, C=C^f*H*-N), 7.36-7.34 (3H, m, C^{a,b}*H*$_{ar}$), 7.26-7.24 (2H, m, *C*^{c}*H*$_{ar}$), 5.50 (2H, s, *C*^{e}*H*$_{2Bz}$), 3.69 (2H, s, *C*^{h}*H*$_2$-N).

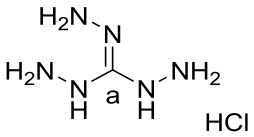
^{13}C NMR (125 MHz, $CDCl_3$): δ_C 144.0 (*qC*), 134.5 (*qC*), 128.8 (2 × *C*H) 128.4 (*C*H), 127.7 (2 × *C*H), 123.5 (*C*H), 53.8 (CH_2), 46.8 (CH_2).

HPLC (method 1): t_R = 8.8 min (99% purity, ELSD)

MS (CI) *m/z*: 553.0 [$(M+Na)^+$, 100%], 531.1 [$(M+H)^+$, 32%]

R_f (DCM/methanol 95:5): 0.90

7.3.7. Triaminoguanidine monohydrochloride (3.22)



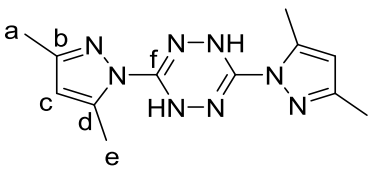
Hydrazine monohydrate (16 mL, 340 mmol, 3.2 eq) was added to a well-stirred slurry of guanidine hydrochloride (10.3 g, 108 mmol, 1 eq) in 1,4-dioxane (50 mL), the reaction mixture was refluxed for 2 h. On cooling the reaction, a white solid formed, which was collected by filtration, washed with 1,4-dioxane, and dried under vacuum to yield the desired product (15.08 g, 107 mmol, 99%).[106,143]

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 159.5 ($C^a(N)_3$).

MS (ESI) *m/z*: 105.2 [$(M-Cl)^+$, 100%]

HPLC (method 2): t_R = 0.6 min (99% purity, ELSD)

7.3.8. 3,6-Bis(3,5-dimethylpyrazol-1-yl)-1,2-dihydro-1,2,4,5-tetrazine (3.23)



To a solution of triaminoguanidine monohydrochloride **3.22** (7.08 g, 50.1 mmol, 1 eq) in water (50 mL), 2,4-pentanedione (10.2 mL, 100.2 mmol, 2 eq) was added dropwise over 1 h, the mixture stirred for a further 30 min at RT, and the reaction heated to 70 ºC for 4 h. The yellow solid, which precipitated from the cooled mixture was filtered, washed with water and dried to yield the product (9.40 g, 34.5 mmol, 69%) as a yellow solid.[106,143]

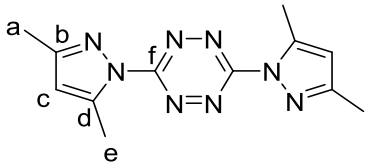
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.07 (2H, bs, N*H*), 5.98 (2H, s, C^c*H*), 2.50 (6H, s, C^a*H*$_3$), and 2.23 (6H, s, C^e*H*$_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 150.0 (*qC*), 145.8 (*qC*), 142.3 (*qC*), 109.9 (*C*H), 13.8 (*C*H$_3$), and 13.5 (*C*H$_3$).

HPLC (method 1): t_R = 9.3 min (97% purity, ELSD)

MS (ESI) *m/z:* 271.2 [(M-H)$^+$, 18%],

7.3.9. 3,6-Dihydrazino-1,2,4,5-tetrazine (3.20)



A solution of $NaNO_2$ (2.88 g, 41.7 mmol, 2.8 eq) in water/DCM (9:1, 72 mL) was added to 3,6-bis(3,5-dimethylpyrazol-1-yl)-1,2-dihydro-1,2,4,5-tetrazine **3.23** (4.0 g, 14.7 mmol, 1 eq), and cooled to 0 °C. Acetic acid (2.0 mL) was added drop-wise, and the reaction mixture was warmed to room temperature overnight. The organic layer was separated and the aqueous layer was washed with DCM (3 × 50 mL). The combined organic layers were neutralised with 5% *aq*. K_2CO_3, dried (anhyd. $MgSO_4$), filtered, and evaporated to yield the product (2.25 g, 8.3 mmol, 56%) as a red solid.[106]

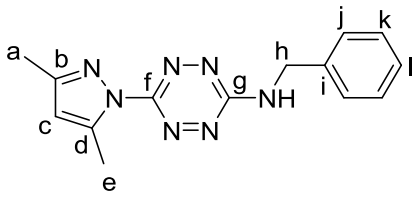
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 6.21 (2H, s, C^{c}*H*), 2.73 (6H, s, C^{a}*H*$_3$), and 2.41 (6H, s, C^{e}*H*$_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 159.6 (*qC*), 154.8 (*qC*), 144.1 (*qC*), 112.2 (*C*H), 15.0 (*C*H$_3$), and 14.1 (*C*H$_3$).

HPLC (method 2): t_R = 8.3 min (95% purity, ELSD)

MS (ESI) *m/z:* 293.2 [$(M+Na)^+$, 53%], 563.2 [$(2M+Na)^+$, 100%]

7.3.10. 3,6-Hydrazinobenzylamine-1,2,4,5-tetrazine (3.26)



To a solution of 3,6-dihydrazino-1,2,4,5-tetrazine **3.20** (559 mg, 2.1 mmol, 1 eq) in DCM (10 mL) was added benzylamine (500 µL, 4.5 mmol, 2.1 eq), and the reaction mixture heated at 70 °C for 45 min in a microwave. The crude material was directly purified by column chromatography (DCM/EtOAc, 10:1) to yield the product (472 mg, 1.7 mmol, 80%) as a red solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.43-7.34 (5H, m, *C*$^{j\text{-}l}$*H*$_{Ar}$), 6.12 (1H, s, C^{c}*H*), 2.58 (3H, s, C^{a}*H*$_3$), 2.37 (3H, s, C^{e}*H*$_3$), and 1.65 (2H, s, C^{h}*H*$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 161.3 (*qC*), 157.9 (*qC*), 152.1 (*qC*), 142.1 (*qC*), 136.8 (*qC*), 129.0 (2 × *C*H), 128.1 (*C*H), 127.9 (2 × *C*H), 109.8 (*C*H), 45.7 (*C*H$_2$), 13.7 (*C*H$_3$), and 13.6 (*C*H$_3$).

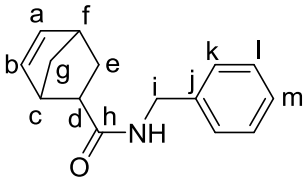
HPLC (method 1): t_R = 6.7 min (93% purity, ELSD)

MS (ESI) *m/z:* 282.1 [$(M+H)^+$, 100%]

R_f (DCM/ethyl acetate, 10:1): 0.21

MP: 149-151 °C

7.3.11. N-Benzylbicyclo[2.2.1]hept-5-ene-2-carboxamide (3.25)



exo-5-Norbornenecarboxylic acid (594 mg, 4.3 mmol, 1 eq), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (850 mg, 4.4 mmol, 1.02 eq) and triethylamine (1.2 mL) were dissolved in DCM (10 mL). After 10 min, benzylamine (800 µL, 7.3 mmol, 1.7 eq) was added to the activated carboxylic acid solution and the reaction mixture was microwave heated at 75 °C for 30 min. The product was isolated directly by column chromatography (10% ethyl acetate in DCM) to yield the product as a white solid (780 mg, 3.4 mmol, 79%).

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.37-7.34 (2H, m, C^k*H*$_{Ar}$), 7.31-7.29 (3H, m, C^{l,m}*H*$_{Ar}$), 6.17-6.09 (2H, m, C^a*H*=C^b*H*), 2.99-2.97 (1H, m, C^f*H*), 2.95-2.92 (1H, m, C^c*H*), 2.04-2.02 (1H, m, C^e*H*$_2$), 1.99-1.96 (1H, m, C^e*H*$_2$), 1.78-1.76 (1H, m, C^d*H*), 1.57 (2H, bs, NHC^i*H*$_2$), 1.40-1.37 (1H, m, C^g*H*$_2$), and 1.36-1.33 (1H, m, C^g*H*$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 175.4 (*C*(O)NH), 138.6 (*qC*), 138.3 (2 × *C*H), 136.0 (2 × *C*H), 128.7 (*C*H=CH), 127.8 (*C*H=CH), 127.5 (*C*H), 47.2 (*C*H), 46.4 (*C*H$_2$), 44.8 (*C*H), 43.8 (*C*H$_2$), 41.6 (*C*H), and 30.6 (*C*H$_2$).

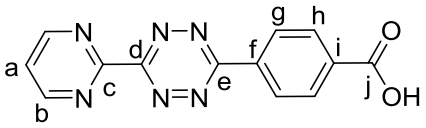
HPLC (method 1): t_R = 7.2 min (95% purity, ELSD)

MS (ESI) *m/z*: 228.1 [(M+H)$^+$, 100%]

R_f (DCM:ethyl acetate, 9:1): 0.52

MP: 118-119 °C

7.3.12. 4-(6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic Acid (3.31)



4-Cyanobenzoic acid (7.0 g, 48 mmol, 1.0 eq) and pyrimidin-2-carbonitrile (5 g, 48 mmol, 1.0 eq) were suspended in ethanol (20 mL), and hydrazine monohydrate (11.5 mL, 238 mmol, 5.0 eq) was added dropwise and the mixture was refluxed overnight. After cooling, the precipitate was removed by filtration and washed with small volumes of ethanol. To remove the bis(pyrimidin-2-yl)-1,2,4,5-dihydrotetrazine by-product, the solid was stirred in acetone (30 mL) and filtered. This was repeated. The filtered solid was suspended in acetic acid (115 mL) and oxidised by the slow addition of isopentyl nitrite (4.5 mL, 33 mmol, 1.5 eq). After stirring overnight, diethyl ether (190 mL) was added to precipitate the product. After filtration, the crude product was purified by flash chromatography (1% acetic acid in hexane:DCM 3:7, followed by 1% acetic acid in DCM, followed by 10% methanol and 1% acetic acid in DCM) to yield 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl) benzoic acid (1.8 g, 6.4 mmol, 13%) as a purple solid.[107]

^{1}H NMR (500 MHz, DMSO): δ_H 9.21 (2H, d, J = 4.9 Hz, C$^{b}H_{Ar}$), 8.27-8.24 (2H, m, C$^{g}H_{Ar}$), 8.19-8.16 (2H, m, C$^{h}H_{Ar}$), and 7.84 (1H, d, J = 4.9 Hz, C$^{a}H_{Ar}$).

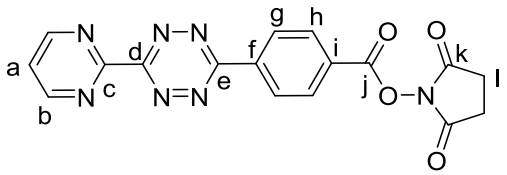
^{13}C NMR (125 MHz, CDCl$_3$): δ_C 166.6 (*C*(O)O), 163.1 (*C*NN), 162.8 (*C*NN), 159.0 (*qC*), 158.4 (*C*H$_{Ar}$), 132.5 (*qC*), 130.2 (2 × *C*H), 129.8 (*qC*), 128.3 (2 × *C*H), and 122.3 (*C*H).

HPLC (method 2): t_R = 6.7 min (99% purity, ELSD)

MS (ESI) *m/z*:279.1 [(M-H)$^-$, 100%]

R_f (DCM/methanol/acetic acid 94:5:1): 0.11

7.3.13. 2,5-Dioxopyrrolidin-1-yl 6-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoate



4-(6-(Pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid **3.31** (605 mg, 2.14 mmol, 1 eq) was suspended in DMF/pyridine (19:1, 25 mL) before the addition of *N*-hydroxysuccinimide (370 mg, 3.21 mmol, 1.5 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl, 500 mg, 3.22 mmol, 1.5 eq). The mixture was heated to 40 °C to give a homogeneous solution and after 2 h, the solvent was removed under reduced pressure. The crude residue was re-dissolved in DCM (50 mL) and washed with water (50 mL). The aqueous layer was extracted with DCM (20 mL). The combined organic layers were dried with anhyd. $MgSO_4$, filtered and evaporated. The residue was dissolved in DCM (40 mL) and precipitated by the addition of diethyl ether (approx. 300 mL). The precipitate was collected by filtration and washed with diethyl ether. After removal of residual solvent *in vacuo*, 2,5-dioxopyrrolidin-1-yl 6-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoate (527 mg, 1.39 mmol, 65%) was isolated as a red solid.[107]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 9.18 (2H, d, *J* = 4.9 Hz, C^b*H*$_{Ar}$), 8.94-8.90 (2H, m, C^h*H*$_{Ar}$), 8.43-8.39 (2H, m, C^g*H*$_{Ar}$), 7.63 (1H, d, *J* = 4.9 Hz, C^a*H*$_{Ar}$), and 2.96 (4H, br s, C^l*H*$_2$).

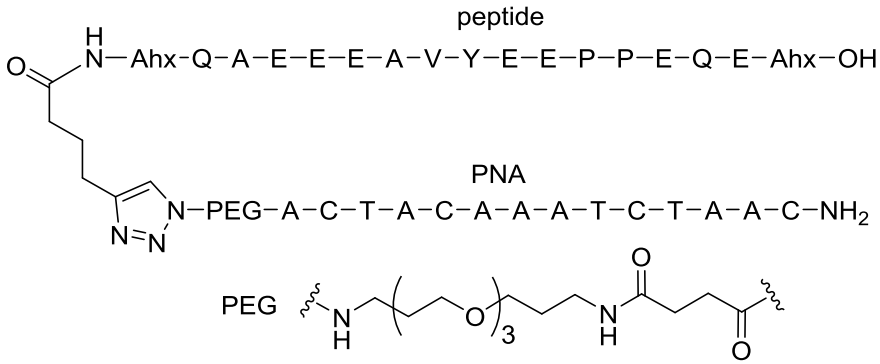
^{13}C NMR (125 MHz, $CDCl_3$): δ_C 168.7 (*C*(O)ON), 163.5 (*C*NN), 163.0 (*C*NN), 161.0 (2 × *C*(O)N), 159.1 (*qC*), 158.2 (*C*H), 136.7 (*qC*), 131.1 (2 × *C*H), 128.9 (*qC*), 128.7 (2 × *C*H), 124.4 (*C*H), and 25.4 (2 × *C*H).

HPLC (method 2): t_R = 5.3 min (99% purity, ELSD)

7.3.14. Ligation of peptides

For the test reactions **Pep035** (**Appendix 2**) was synthesised on 2-chlorotrityl functionalised polystyrene resin pre-loaded with Fmoc-Lys(Dde)-OH (250 mg, 0.072 mmol, **5.3**) using a peptide synthesiser. **PNA100** (**Appendix 1**) which was synthesised on Fmoc-Rink-LL-PS resin was used for the test reactions unless otherwise stated.

7.3.15. Test click reaction with the TBTA ligand (3.10)



Peptide and PNA Synthesis:

The peptide and PNA were prepared using standard peptide couplings (**Section 7.2**) with the peptide and PNA capped with 5-hexynoic acid and 6-azidohexanoic acid **3.3** respectively. The protected peptide was obtained as a white solid (16.4 mg, 65%).

MS (MALDI) *m/z*: calc. 3468.92, experimental 3494.93 $[M+Na]^+$ [48%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

Ligation:

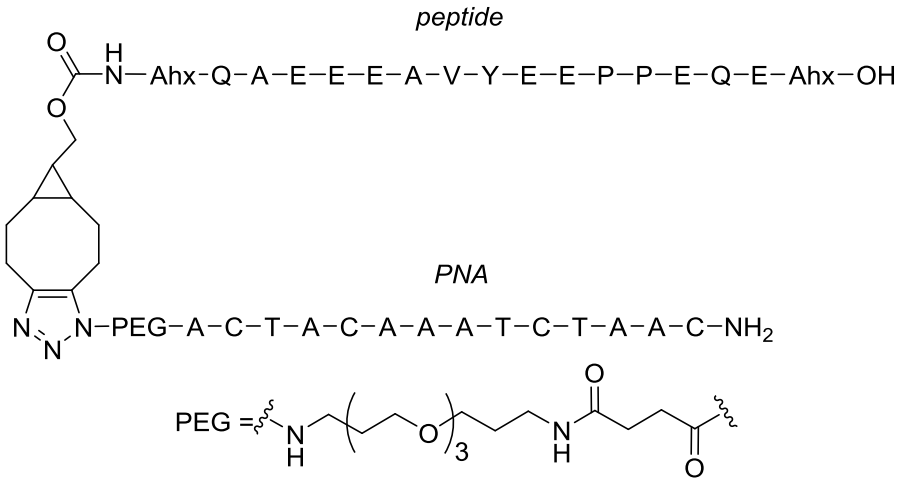
A solution of CuI (0.6 mg, 0.003 mmol, 3 eq), sodium ascorbate (1.8 mg, 0.009 mmol, 9 eq) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine **1.8** (3 mg, 0.006 mmol, 6 eq) in 20% piperidine in DMF (200 μL) was prepared and added to a solution of the peptide (10 mg, 0.003 mmol, 3 eq) in DMF/DMSO (1:1, 300 μL).

This solution was added to the azide-PNA resin and the reaction was shaken overnight. The resin was washed with DMSO (5 × 0.5 mL), 20% piperidine in DMF (3 × 0.5 mL), DMF (3 × 0.5 mL), DCM (3 × 0.5 mL) and methanol (3 × 0.5 mL). The conjugate was cleaved from the resin using the method described in **Section 7.2** (TFA:TIS:DCM), analysed by HPLC and MALDI, and after purification with HPLC (Method 4) a white solid was obtained (2.5 mg, 0.395 μmol, 40%). ICP-MS (Agilent 7500ce with an octopole reaction system) analysis of the conjugate (1.25 mg sample) showed a copper concentration of 1.57–1.82 ppb (0.025–0.029 μM).

HPLC (method 4): t_R = 14.3 min (38% purity of crude, 254 nm)

MS (MALDI) ***m/z****:* calc. 6330.95, experimental 6336.63 [100%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

7.3.16. Test "copper free click" ligation (3.16)



Peptide and PNA Synthesis:

The peptide and PNA (synthesised in **Section 7.3.15**) were capped with bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate and 6-azidohexanoic acid, respectively. The protected peptide was obtained as a white solid (19.1 mg, 74%).

MS (MALDI) ***m/z:*** calc. 3550.96, experimental 3576.91 $[M+Na]^+$ [23%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

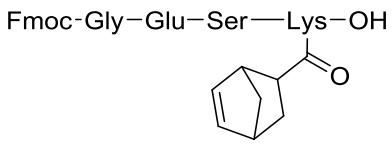
Ligation:

The ligation was carried out by adding a solution of the peptide (10 mg, 0.003 mmol, 3 eq) in DMF/DMSO (1:2, 450 μL) to the azide-PNA resin with shaking overnight. The resin was washed with DMSO (5 × 0.5 mL), DMF (3 × 0.5 mL), DCM (3 × 0.5 mL) and methanol (3 × 0.5 mL) and the conjugate was cleaved from the resin using the method described in **Section 7.2.5**. The conjugate was analysed by HPLC and MALDI and no purification was undertaken.

HPLC (method 4): t_R = 14.2 min (28% purity, 254 nm)

MS (MALDI) ***m/z:*** calc. 6412.99, experimental 6417.34 [100%], calc. 4186.79 (azide-PNA starting material), experimental 4191.56 [18%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

7.3.17. Test tetrazine ligation with 5-Norbornene-2-carboxylic



Activation of the chlorotrityl linker: 2-Chlorotrityl-polystyrene resin (100 mg, 0.14 mmol) in a solid phase extraction tube was swollen in dry DCM (2 mL) for 30 min, filtered, and a solution of thionyl chloride (50 μL, 0.68 mmol, 4.5 eq) in dry DCM (2 mL) was added, mixture shaken for 1 h, filtered, and washed with dry DCM (3 × 2 mL).[144]

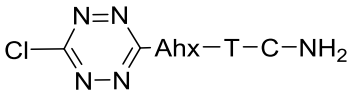
First acid coupling onto activated chlorotrityl linker: A solution of Fmoc-Lys(Dde)-OH (**4.5**, 149 mg, 0.28 mmol, 2 eq) and DIPEA (14 μL, 0.15 mmol, 1.1 eq) in dry

DCM (2 mL) was added and the resin was shaken for 30 min. After washing with DMF (3 × 2 mL), DCM (3 × 2 mL) and methanol (2 × 2 mL), unreacted chlorides were displaced by adding a solution of DCM, methanol and DIPEA (80:15:5, 2 × 2 mL) and further shaking for 15 min.

The peptide was synthesised following the Fmoc-deprotection and coupling procedures (**Section 7.2**), the Dde group was deprotected and a mixture of *endo*- and *exo*-norbornene-2-carboxylic acid (66 μL, 0.42 mmol, 3 eq) were coupled using the method described in **Section 7.2**. Finally, the norbornene modified peptide was cleaved by treatment of the linker with acetic acid, trifluoroethanol and DCM (2:2:6, 2 mL) for 2 h, and precipitation in cold ether to afford a white solid (6.2 mg, 0.007 mmol, 10%).

HPLC (method 2): t_R = 8.3/8.4 min (75% purity, 254 nm, isomer of norbornene)

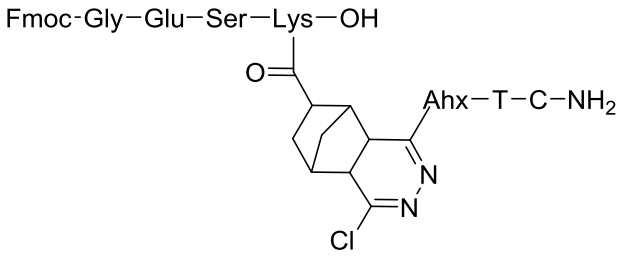
MS (ESI) *m/z*: calc. 873.45, experimental 896.2 [$(M+Na)^+$, 100%].



Fmoc-Rink-LLPS (100 mg, 0.018 mmol) was placed in a solid phase extraction tube and swollen for 30 min in DCM. The PNA sequence was synthesised following standard Fmoc-chemistry (**Section 7.2**) and capped with an Fmoc-aminohexanoic acid. After Fmoc-deprotection, a solution of dichloro-tetrazine (8 mg, 0.054 mmol, 3 eq) and DIPEA (2 μL, 0.019 mmol, 1.05 eq) in DCM (2 mL) was added and the reaction was agitated for 2 h. Completion of the reaction was confirmed by a Ninhydrin test. A small sample was cleaved, purified and analysed.

HPLC (method 3): t_R = 6.5 min (99% purity, 254 nm)

MS (MALDI) *m/z*: calc. 761.29, experimental 763.1, 765.2 [$(M+H)^+$, 100%, 3:1]

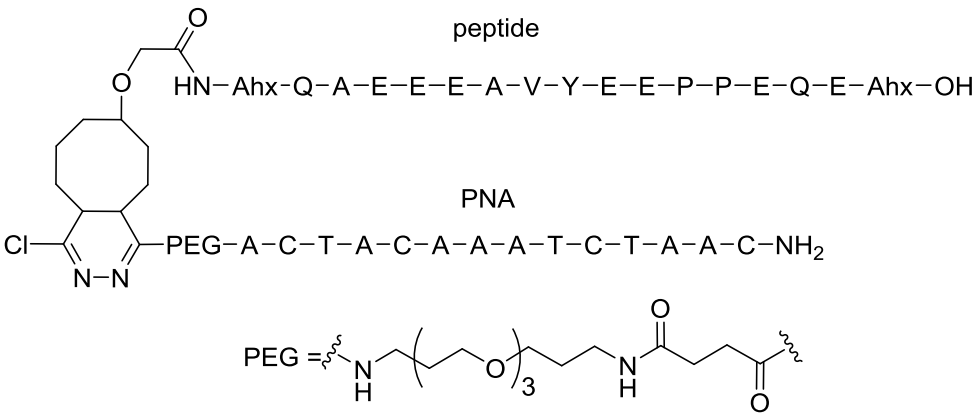


The crude peptide (synthesised above, 3.5 mg, 0.004 mmol, 1.3 eq) was dissolved in a solution of DMF/DMSO (1:1, 0.5 mL), added to the tetrazine-PNA resin (20 mg, 0.0036 mmol) and the reaction was shaken at 75 °C overnight. The resin was washed with DMSO (3 × 0.7 mL), DMF (3 × 0.7 mL), DCM (3 × 0.7 mL) and methanol (3 × 0.7 mL). The conjugate was cleaved and analysed by MALDI and HPLC.

HPLC (method 2): t_R = 3.6 min (29% purity, 254 nm)

MS (MALDI) ***m/z****:* calc. 1494.61, experimental 1495.9 [11%], 764.5 [100%] (unreacted PNA starting material).

7.3.18. Test tetrazine ligation with (E)-cyclooct-4-yl 2,5-dioxopyrrolidin-1-yl carbonate (3.30)



Deprotection and coupling procedures are described in **Section 7.2**.

To **Pep035** (**Appendix 2**, 30 mg, theoretical loading 0.0073 mmol) was coupled Fmoc-aminohexanoic acid. After the final deprotection, a solution of (*E*)-cycloocten-4-yl-2,5-dioxopyrrolidin-1-yl carbonate (6.0 mg, 0.022 mmol, 3 eq) in DMF was added and the reaction left shaking overnight. Completion of the reaction was confirmed by the ninhydrin test. After washing the resin, the peptide was cleaved with a solution of acetic acid, trifluoroethanol and DCM (2:2:6, 0.5 mL) for 3 × 1 h, and precipitated from cold ether (19.2 mg, 75%).

MS (MALDI) *m/z*: calc. 3526.96, experimental 3552.97 $[M+Na]^+$ [30%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

To **PNA100** (10 mg, 0.001 mmol) was coupled Fmoc-PEG-OH (2 mg, 0.003 mmol, 3 eq) with Oxyma as a coupling agent. After Fmoc-deprotection, a solution of dichloro-tetrazine (0.5 mg, 0.003 mmol, 3 eq) and DIPEA (1 μL, 0.006 mmol, 6 eq) in DCM (0.5 mL) was added to the resin and the mixture shaken for 3 h. After washing the resin, the reaction completion was confirmed by ninhydrin test and the resin was used in the next step without further analysis.

Ligation:

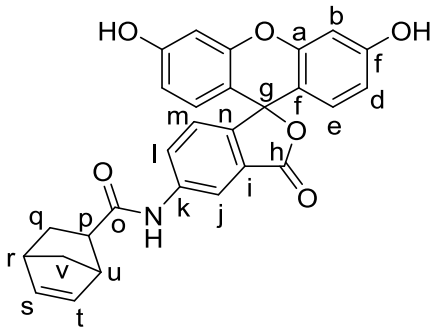
The ligation was carried out by adding a solution of the peptide synthesised above (10 mg, 0.003 mmol, 3 eq) in DMF/DMSO (1:2, 450 μL) to the tetrazine-PNA resin and the reaction shaken overnight. The resin was washed with DMSO (5 × 0.5 mL), DMF (3 × 0.5 mL), DCM (3 × 0.5 mL) and methanol (3 × 0.5 mL). The PNA-peptide conjugate was cleaved from the resin using method described in **Section 7.2.5** and analysed by HPLC and MALDI.

HPLC (method 4): t_R = 14.3 min (33% purity, 254 nm)

MS (MALDI) *m/z*: calc. 6335.89, experimental 6341.53 [36%] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

7.4. Chapter 4

7.4.1. N-*(exo-5-Norbornene)-5-fluorescein-carboxamide (4.3)*



Norbornene-2-carboxylic acid (41 mg, 0.3 mmol, 1 eq) and HBTU (110 mg, 0.29 mmol, 0.97 eq) where dissolved in DMF (2 mL) and DIPEA (104 µL, 0.6 mmol, 2 eq) was added to the solution, and stirred for 10 min. To the activated carboxylic acid, 5-aminofluorescein (104.2 mg, 0.3 mmol, 1 eq) was added, and the reaction was microwave heated at 75 °C for 45 min. After solvent evaporation, the crude solid was dissolved in a solution of NaOH (72 mg, 1.8 mmol, 6 eq) in water and THF (1:1, 5 mL) and stirred overnight at 60 °C. The reaction was acidified with 1N HCl and the precipitate was filtered of, and purified by prep-HPLC to yield the product (17 mg, 0.04 mmol, 13%) as a yellow solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 10.44 (2H, bs, Ar-O*H*), 8.32 (1H, d, *J* = 1.6 Hz, C^j*H*$_{Ar}$), 7.84 (1H, dd, *J* = 8.3, 1.6 Hz, C^l*H*$_{Ar}$), 7.18 (1H, d, *J* = 8.3 Hz, C^m*H*$_{Ar}$), 6.63 (2H, bs, C^b*H*$_{Ar}$), 6.60 (2H, d, *J* = 8.7 Hz, C^e*H*$_{Ar}$), 6.52 (2H, d, *J* = 8.7 Hz, C^d*H*$_{Ar}$), 6.26-6.18 (2H, m, C^s*H*=C^t*H*), 3.01-2.98 (1H, m, C^r*H*), 2.95-2.91 (1H, m, C^u*H*), 2.35-2.33 (1H, m, C^p*H*), 1.95-1.92 (1H, m, C^q*H*$_2$), 1.69-1.67 (1H, m, C^q*H*$_2$), 1.33-1.29 (1H, m, C^v*H*$_2$), and 1.27-1.24 (1H, m, C^v*H*$_2$).

HPLC (method 2): t_R = 12.8 min (99% purity, ELSD)

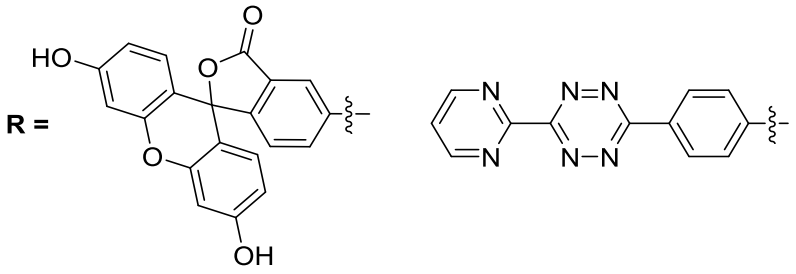
MS (ESI) *m/z:* 468.2 [$(M+H)^+$, 15%]

MP: decomposed >250 °C

7.4.2. Probe-synthesis

7.4.2.1. GAPDH-PNA-Probe (4.1)

R-PEG-G-A-G-T-C-C-T-T-C-C-A-C-G-A-T-A-C-C-A-NH_2



A **B**

Synthesis of the two probes was performed on aminomethyl ChemMatrix resin (20 mg, 20 µmol). Sequential coupling and deprotection of PNA monomers are described in **Section 7.2** After the PNA synthesis, a PEG spacer (46 mg, 0.12 mmol, 3 eq), followed by coupling of 5(6)-carboxylfluorescein (45 mg, 0.12 mmol, 3 eq, **GAPDH-A**) or 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (**3.31**, 33 mg, 0.12 mmol, 3 eq, **GAPDH-B**) using Oxyma/DIC for 2 × 1 h. The probe was cleaved as described in **Section 7.2.5**. After HPLC-purification the product was collected as a yellow solid (8 mg, 1.4 µmol, 7%) for **GAPDH-A** and a red solid (7 mg, 1.3 mmol, 7%) for **GAPDH-B**.

***GAPDH-A**:*

HPLC (method 4): t_R = 10.1 min (99% purity, 254 nm)

MS (MALDI) *m/z*: calc. 5593.17, experimental 5593.40 [100%, $M-H^-$].

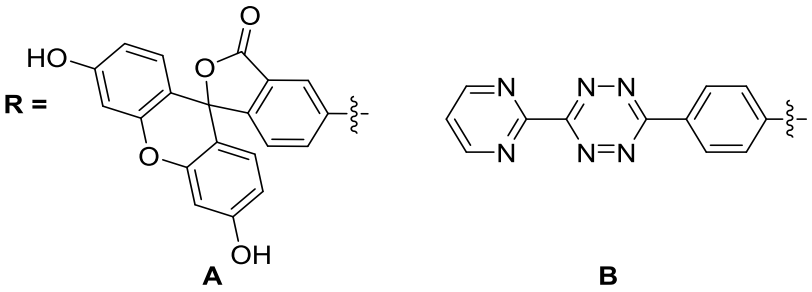
***GAPDH-B**:*

HPLC (method 4): t_R = 9.6 min (97% purity, 254 nm)

MS (MALDI) *m/z*: calc. 5495.17, experimental 5498.10 [100%, $M+H^+$] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

7.4.2.2. 28S rRNA-PNA-Probe (4.2)

R-PEG-A-C-C-A-C-C-A-A-G-A-T-C-T-G-C-NH_2



The synthesis and quantities were as in **Section 7.4.2.1**. After HPLC-purification the product was collected as a yellow solid (12.5 mg, 2.8 µmol, 14%) for **28S rRNA-A** and a red solid (5.9 mg, 1.3 mmol, 7%) for **28S rRNA-B**.

***28S rRNA-A**:*

HPLC (method 4): t_R = 10.2 min (99% purity, 254 nm)

MS (MALDI) *m/z*: calc. 4516.74, experimental 4518.58 [100%, $M+H^+$] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

***28S rRNA-B**:*

HPLC (method 4): t_R = 9.6 min (99% purity, 254 nm)

MS (MALDI) *m/z*: calc. 4420.76, experimental 4422.47 [100%, $M+H^+$] (error in mass, see internal standard test **Chapter 5**, **Figure 5.2**).

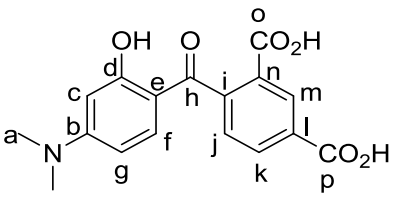
7.4.2.3. Fluorescence In-Situ Hybridisation (FISH)

Hela cells (200,000 per well) were fixed in a 96-well PS plate using 4% *aq* para formaldehyde for 20 min, washed with PBS (2 × 10 min, pH 6.5) and incubated with the probes (**28S rRNA** and **GAPDH**, 4 µM) at 37 °C for 20 h. The cells were washed with PBS (2 × 5 min, pH 6.5), followed by the addition of Flu-Nor (**4.3**, 4

µM) to the TZ-PNA probes. Which were incubated for a further 2 h. The cells were washed with PBS (2 × 5 min, pH 6.5), nucleus stained with DAPI (1 × 30 min), followed by washing with PBS (2 × 5 min, pH 6.5) and finally imaged by semi-confocal microscopy.

7.5. Chapter 5

7.5.1. 4-Dimethylamino-2-hydroxy-2',4'-dicarboxy-benzophenone (5.6)



3-Dimethylaminophenol (13.7 g, 0.10 mol) was dissolved in toluene (300 mL), heated to 60 °C, and trimellitic anhydride (23.0 g, 0.12 mol) added. The mixture was refluxed for 24 h. After cooling to room temperature, the solid was filtered, washed with toluene (3 × 50 mL), dissolved in methanol (300 mL), and refluxed for 10 min. Acetic acid (100 mL) was added and the mixture was evaporated to dryness. The solid was recrystallized from MeOH (200 mL), collected by filtration and washed with methanol (50 mL) to give the product (6.29 g, 19%) as a red crystalline solid.[138]

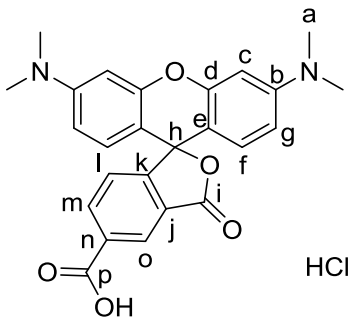
^{1}H NMR (500 MHz, DMSO): δ_H 13.38 (2H, bs, C^{o,p}OO*H*), 12.36 (1H, s, C^{d}O*H*), 8.49 (1H, d, *J* = 1.7 Hz, C$^{m}H_{Ar}$), 8.20 (1H, dd, *J* = 7.8, 1.7 Hz, C$^{k}H_{Ar}$), 7.52 (1H, d, *J* = 7.8 Hz, C$^{j}H_{Ar}$), 6.81 (1H, d, *J* = 9.0 Hz, C$^{f}H_{Ar}$), 6.22 (1H, dd, *J* = 9.0, 2.4 Hz, C$^{g}H_{Ar}$), 6.11 (1H, d, *J* = 2.4 Hz, C$^{c}H_{Ar}$), and 3.01 (6H, s, NC^{a}H$_3$).

^{13}C NMR (125 MHz, DMSO): δ_C 197.6 (*C*(O)), 166.1 (*C*(O)O), 164.2 (*qC*), 155.8 (*qC*), 143.7 (*qC*), 133.8 (*C*H), 132.7 (*C*H), 131.6 (*C*), 130.7 (*C*H), 130.0 (*C*H), 128.3 (*C*H), 109.5 (q*C*), 104.5 (*C*H), 97.0 (*C*H), and 39.5 (*C*H$_3$).

HPLC (method 2): t_R = 8.3 min (99% purity, ELSD)

MS (ESI) *m/z*: 657.2 [(2M-2H)$^-$, 72%]

7.5.2. 5-Carboxytetramethylrhodamine monohydrochloride (TAMRA•HCl, 5.5)



4-Dimethylamino-2-hydroxy-2′,4′-dicarboxybenzophenone **5.6** (3.29 g, 10 mmol) and 3-dimethylaminophenol (1.78 g, 13 mmol) were dissolved in dry DMF (80 mL). Trimethylsilylpolyphosphate (10 mL) in dry DCM (5 mL) was added, and the mixture refluxed for 3 h. Solvents were removed *in vacuo*, the residue dissolved in 5% NaOH (70 mL), and stirred overnight. The solution was diluted with water (150 mL) and lyophilised. The crude product was purified by column chromatography (methanol/acetic acid/DCM, 2:2:96 to 15:5:80). Residual acetic acid was removed from the product by acidifying a solution of 5-TAMRA in water with 1M hydrochloric acid (20 mL, 2.2 eq) and lyophilisation to yield 5-TAMRA•HCl (4.2 g, 90%) as a dark red solid.[138]

^{1}H NMR (500 MHz, CD_3OD): δ_H 8.88 (1H, s, C^oH_{Ar}), 8.4 (1H, d, J = 7.9 Hz, C^mH_{Ar}), 7.52 (1H, d, J = 7.9 Hz, C^lH_{Ar}), 7.10 (2H, d, J = 9.4 Hz, C^fH_{Ar}), 7.04 (2H, d, J = 9.4 Hz, C^gH_{Ar}), 6.95 (2H, s, C^cH_{Ar}), and 3.18 (12H, bs, NC^aH_3).

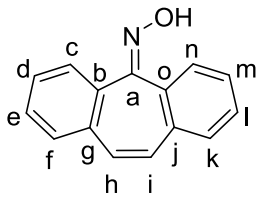
^{13}C NMR (125 MHz, CD_3OD): δ_C 168.3 (*C*(O)O), 168.0 (*C*(O)O), 160.9 (*qC*), 159.1 (*qC*), 158.9 (*qC*), 139.1 (*qC*), 134.5 (*qC*), 134.0 (*C*H), 133.3 (*C*H), 132.0 (*C*H), 131.8 (*C*H), 115.5 (*qC*), 114.7 (*C*H), 97.4 (*C*H), 40.9 (*C*H_3), and 20.7 (*qC*).

HPLC (method 2): t_R = 7.1 min (99% purity, ELSD)

MS (ESI) *m/z:* 431.3 [$(M+H)^+$, 50%]

R_f (1% acetic acid / 15% methanol / DCM): 0.21

*7.5.3. 5*H*-Dibenzo[7]annulene-5-one oxime (5.12)*



Dibenzosuberenone **5.11** (10 g, 48.5 mmol) and hydroxylamine hydrochloride (4.4 g, 63.8 mmol) were dissolved in pyridine (30 mL), and the mixture refluxed for 18 h. The solution was concentrated under reduced pressure, and poured onto crushed ice (300 g) containing 5% hydrochloric acid (200 mL). The product was collected by filtration to yield a pale yellow solid (9.46 g, 42.8 mmol, 88%).[105]

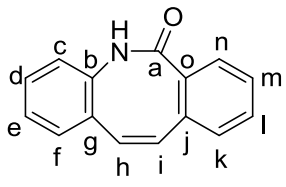
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.68-7.66 (1H, m, C$^{c}H_{Ar}$), 7.61-7.59 (1H, m, C$^{n}H_{Ar}$), 7.46-7.40 (6H, m, C$^{d\text{-}f,k\text{-}m}H_{Ar}$), and 6.93-6.92 (2H, m, C$^{h,j}H$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 157.1 (*C*=NO), 135.4 (*qC*), 134.9 (*qC*), 134.6 (*qC*), 133.6 (*qC*), 131.9 (*C*H), 131.7 (*C*H), 130.7 (*C*H), 130.2 (*C*H), 129.4 (*C*H), 129.0 (*C*H), 128.8 (*C*H), 128.6 (*C*H), 127.6 (*C*H), and 126.8 (*C*H).

HPLC (method 2): t_R = 7.1 min (99% purity, ELSD)

MS (ESI) *m/z:* 222.1 [$(M)^+$, 100%]

*7.5.4. Dibenzo[*b,f*]azocin-6(5*H*)-one (5.10)*



5*H*-Dibenzo[7]annulene-5-one oxime **5.12** (9.46 g, 42.8 mmol) and polyphosphonic acid (100 mL) were heated at 140 °C for 2 h. The reaction mixture was poured onto crushed ice (600 g) and stirred for 30 min. The solid was filtered, washed with water

(3 × 50 mL), and dried under vacuum to yield the desired product as a grey solid (8.89 g, 40.1 mmol, 94%).[105]

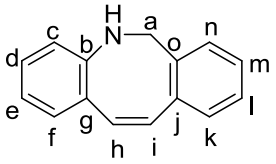
^{1}H NMR (500 MHz, D$_6$-DMSO): **)**: δ_H 7.33-7.30 (2H, m, C^{c,n}*H*$_{Ar}$), 7.26-7.20 (2H, m, C^{d,m}*H*$_{Ar}$), 7.17-7.08 (4H, m, C^{e,f,k,l}*H*$_{Ar}$), 7.00 (1H, d, *J* = 11.6 Hz, C^{h}*H*), and 6.89 (1H, d, *J* = 11.6 Hz, C^{i}*H*).

^{13}C NMR (125 MHz, D$_6$-DMSO): 172.2 (*C*(O)N), 136.7 (*qC*), 136.6 (*qC*), 134.9 (*qC*), 133.9 (*qC*), 133.1 (*C*H), 130.7 (*C*H), 139.4 (*C*H), 129.3 (*C*H), 128.5 (*C*H), 128.3 (*C*H), 128.2 (*C*H), 127.9 (*C*H), 126.9 (*C*H), and 126.6 (*C*H).

HPLC (method 2): t_R = 7.6 min (99% purity, ELSD)

MS (ESI) *m/z*: 222.0 [(M+H)$^+$, 15%], 442.9 [(2M+H))$^+$, 100%]

*7.5.5. 5,6-Dihydrodibenzo[*b,f*]azocine (5.13)*



Dibenzo[*b*,*f*]azocin-6(5*H*)-one **5.10** (8.89 g, 40.1 mmol) and $LiAlH_4$ (3.08 g, 81.2 mmol) were suspended in dry ether (200 mL) and the mixture refluxed for 18 h. The reaction was quenched with water (10 mL), followed by filtration. The filter cake was washed with ether (50 mL), and then dispersed in ether (100 mL). This suspension was stirred for 30 min and re-filtered. The combined organic layers were dried (anhyd. $MgSO_4$), and evaporated to yield the product as a yellow solid (4.5 g, 21.7 mmol, 54%).[105]

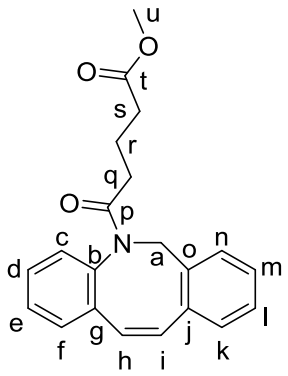
^{1}H NMR (500 MHz, CDCl$_3$): δ_H 7.26-7.24 (1H, m, C^{c}*H*$_{Ar}$), 7.21-7.17 (3H, m, C$^{c-e,l}$*H*$_{Ar}$), 6.99-6.96 (1H, m, C^{f}*H*$_{Ar}$), 6.91-6.88 (1H, m, C^{k}*H*$_{Ar}$), 6.63-6.60 (1H, m, C^{m}*H*$_{Ar}$), 6.55 (1H, d, *J* = 13.2 Hz, C^{h}*H*), 6.49-6.47 (1H, m, C^{n}*H*$_{Ar}$), 6.37 (1H, d, *J* = 13.1 Hz, C^{i}*H*), and 4.59 (2H, s, NC^{a}*H*$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 147.2 (*qC*), 139.2 (*qC*), 138.1 (*qC*), 134.7 (*C*H), 132.7 (*C*H), 130.1 (*C*H), 128.9 (*C*H), 127.9 (*C*H), 127.6 (*C*H), 127.4 (*C*H), 127.3 (*C*H), 121.8 (*qC*), 117.9 (*C*H), 117.8 (*C*H), and 49.6 (CH_2).

HPLC (method 2): t_R = 7.6 min (68% crude purity, ELSD)

MS (ESI) *m/z*: 208.0 [$(M+H)^+$, 100%]

7.5.6. Methyl-5-dibenzo[b,f]azocin-5(6H)-yl-5-oxopentanoate (5.14)



A solution of 5,6-dihydrodibenzo[*b,f*]azocine **5.13** (4.5g, 21.7 mmol), glutaric acid monomethyl ester chloride (4.5g, 27.3 mmol) and pyridine (5 mL, 61.9 mmol) in DCM (50 mL) were stirred for 30 min. Additional DCM (50 mL) was added to the reaction mixture and the organic layer washed with water (2 × 150 mL). The organic layer was dried (anhyd. $MgSO_4$), evaporated, and the residue purified by column chromatography (hexane/EtOAc 2:1) to yield the desired product (3.98 g, 11.9 mmol, 55%) as a yellow oil.[105]

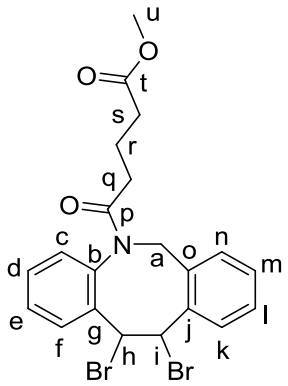
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.29-7.24 (4H, m, $C^{c\text{-}f}H_{Ar}$), 7.18-7.12 (4H, m, $C^{k\text{-}n}H_{Ar}$), 6.78 (1H, d, *J* = 13.1 Hz, C^hH), 6.58 (1H, d, *J* = 13.1 Hz, C^iH), 5.50 (1H, d, *J* = 15.0 Hz, C^aH_2), 4.22 (1H, d, *J* = 15.0 Hz, C^aH_2), 3.58 (3H, s, OC^uH_3), 2.20-2.06 (3H, m, $C^{q\text{-}s}H_2$) and 1.93-1.78 (3H, m, $C^{q\text{-}s}H_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 173.6 (*qC*(O)O), 171.8 (*qC*(O)N), 141.0 (*qC*), 136.3 (*qC*), 135.7 (*qC*), 134.8 (*qC*), 132.6 (*C*H), 132.0 (*C*H), 131.1 (*C*H), 130.3 (*C*H), 128.4 (*C*H), 128.1 (*C*H), 127.9 (*C*H), 127.3 (*C*H), 127.2 (*C*H), 127.2 (*C*H), 127.0 (*C*H), 54.5 (CH_2), 51.4 (CH_3), 33.4 (CH_2), 33.0 (CH_2), and 20.4 (CH_2).

HPLC (method 2): t_R = 9.3 min (99% purity, ELSD)

MS (ESI) *m/z*: 336.0 [$(M+H)^+$, 100%]

7.5.7. Methyl-5-(11,12-dibromo-11,12-dihydrodibenzo[b,f]azocin-5(6H)-yl)-5-oxopentanoate



Bromine (0.63 mL, 12.4 mmol) was added to a solution of methyl-5-dibenzo[*b,f*]azocin-5(6*H*)-yl-5-oxopentanoate **5.14** (3.98 g, 11.9 mmol) in dry DCM (150 mL) over 10 min at 0 °C. The reaction was stirred at 0 °C for 45 min, quenched with sat. Na_2SO_3 (3 × 80 mL), washed with brine (150 mL), and the organic layer dried (anhyd. $MgSO_4$). The product was purified by column chromatography (hexane/ EtOAc 3:1 to 2:1) to yield the desired product (4.23 g, 8.58 mmol, 72 %) as a white solid.[105]

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.73 (1H, d, *J* = 7.7 Hz, C^f*H*), 7.25-7.13 (3H, m, C$^{c\text{-}e}$*H*$_{Ar}$), 7.07-6.98 (3H, m, C$^{l\text{-}n}$*H*$_{Ar}$), 6.89 (1H, d, *J* = 7.6 Hz, C^k*H*), 5.92 (1H, d, *J* = 9.9 Hz, C^h*H*), 5.83 (1H, d, *J* = 14.8 Hz, C^a*H*$_2$), 5.16 (1H, d, *J* = 9.9 Hz, C^i*H*$_2$), 4.17

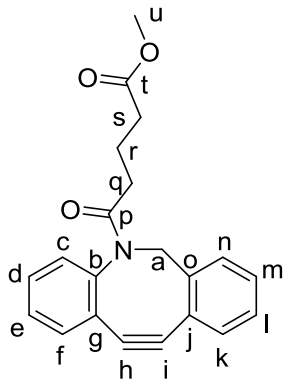
(1H, d, J = 14.8 Hz, C$^{a}H_2$), 3.62 (3H, s, OC$^{u}H_3$), 2.44-2.30 (3H, m, C$^{q,s}H_2$), 2.23-2.17 (1H, m, C$^{q,s}H_2$) and 2.07-1.96 (2H, m, C$^{r}H_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 173.7 (*qC*(O)O), 172.7 (*qC*(O)N), 138.2 (*qC*), 137.2 (*qC*), 137.1 (*qC*), 132.9 (*qC*), 130.7 (*C*H), 130.5 (2 × *C*H), 129.6 (*C*H), 129.4 (*C*H), 128.9 (*C*H), 128.9 (*C*H), 128.6 (*C*H), 60.1 (*C*H), 55.6 (*C*H), 52.5 (*C*H_2), 51.5 (*C*H_3), 34.9 (*C*H_2), 33.3 (*C*H_2), and 20.4 (*C*H_2).

HPLC (method 2): t_R = 9.9 min (99% purity, ELSD)

MS (ESI) *m/z*: 495.8 [$(M+H)^+$, 100%]

*7.5.8. Methyl-5-(11,12-didehydrodibenzo[*b,f*]azocin-5(6*H*)-yl)-5-oxopentanoate (5.15)*



Methyl-5-(11,12-dibromo-11,12-dihydrodibenzo[b,f]azocin-5(6H)-yl-5-oxopentanoate (2.05 g, 4.16 mmol) was dissolved in dry THF (140 mL) and cooled to -40 °C under nitrogen. A solution of KO^{t}Bu (1.8 g, 16.0 mmol) in dry THF (16 mL) was added dropwise over 30 min. The reaction was stirred at -40 °C for 90 min, followed by the addition of additional KO^{t}Bu (0.5 g, 4.5 mmol) in dry THF (5 mL), and stirred for 30 min. The reaction was poured into water (210 mL), and the crude product was extracted with DCM (2 × 280 mL), the combined organic layers washed with water (3 × 350 mL), dried (anhyd. $MgSO_4$) and evaporated. The product was

purified by column chromatography (hexane/ EtOAc 2:1) to yield the desired product (1.02 g, 3.06 mmol, 74%) as a yellow oil.[105]

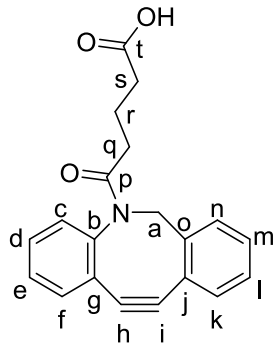
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.72-7.69 (1H, m, C^f*H*), 7.43-7.25 (7H, m, C$^{c\text{-}e,k\text{-}n}$*H*$_{Ar}$), 5.16 (1H, d, *J* = 13.9 Hz, C^a*H*$_2$), 3.60 (1H, d, *J* = 13.9 Hz, C^a*H*$_2$), 3.56 (3H, s, OC^u*H*$_3$), 2.46-2.41 (2H, m, C^s*H*$_2$), 1.99-1.94 (2H, m, C^p*H*$_2$) and 1.78-1.71 (2H, m, C^q*H*$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 173.5 (*qC*(O)O), 172.7 (*qC*(O)N), 151.7 (*qC*), 148.0 (*qC*), 132.9 (*qC*), 132.3 (*C*H), 128.9 (*C*H), 128.3 (*C*H), 128.3 (*C*H), 128.0 (*C*H), 127.7 (*C*H), 127.0 (*C*H), 125.5 (*C*H), 123.0 (*qC*), 115.1 (*qC*), 107.7 (*qC*), 55.3 (*C*H$_2$), 51.4 (*C*H$_3$), 33.7 (*C*H$_2$), 32.8 (*C*H$_2$), and 20.6 (*C*H$_2$).

HPLC (method 2): t_R = 10.4 min (99% purity, ELSD)

MS (ESI) *m/z*: 334.0 [$(M+H)^+$, 100%]

7.5.9. 5-(11,12-Didehydrodibenzo[b,f]azocin-5(6H)-yl)-5-oxopentanoic acid (ADIBO, 5.9)



LiOH (0.272 g, 11.3 mmol) in water (15 mL) was added drop-wise over 15 min to methyl-5-(11,12-didehydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-5-oxopentanoate **5.15** (1.02 g, 3.06 mmol) in THF (45 mL), and the reaction mixture stirred for 4 h. Water (150 mL) was added and the mixture basified using 1 M NaOH. The aqueous layer was washed with DCM (3 × 300 mL), acidified with 1 M HCl, and extracted with DCM

(3 × 200 mL). The combined organic layers were washed with brine (400 mL), dried (anhyd. $MgSO_4$) and evaporated to yield the desired product (0.875 g, 2.74 mmol, 90%) as a pale yellow solid.[105]

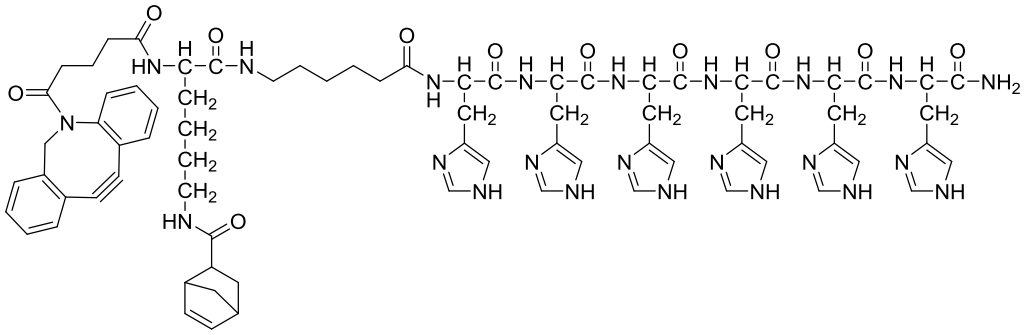
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.69-7.67 (1H, m, C^f*H*), 7.46-7.44 (3H, m, C$^{c\text{-}e}$*H*$_{Ar}$), 7.26-7.23 (4H, m, C$^{l\text{-}n}$*H*$_{Ar}$), 5.29 (1H, d, *J* = 14.3 Hz, C^a*H*$_2$), 3.67 (1H, d, *J* = 14.3 Hz, C^a*H*$_2$), 2.37-2.26 (1H, m, C^s*H*$_2$), 2.19-2.11 (2H, m, C^q*H*$_2$), 2.03-1.98 (1H, m, C^s*H*$_2$), and 1.78-1.6.8 (2H, m, C^r*H*$_2$).

^{13}C NMR (125 MHz, $CDCl_3$): δ_C 178.5 (*qC*(O)O), 172.5 (*qC*(O)N), 151.5 (*qC*), 148.1 (*qC*), 133.4 (*qC*), 132.3 (*C*H), 131.5 (*C*H), 131.1 (*C*H), 130.6 (*C*H), 129.4 (*C*H), 128.4 (*C*H), 127.5 (*C*H), 127.3 (*C*H), 124.4 (*qC*), 115.8 (*qC*), 108.5 (*qC*), 55.2 (*C*H$_2$), 33.0 (*C*H$_2$), 32.8 (*C*H$_2$), and 20.2 (*C*H$_2$).

HPLC (method 2): t_R = 9.6 min (99% purity, ELSD)

MS (ESI) *m/z*: 319.9 [$(M+H)^+$, 100%]

7.5.10. ADIBO-Lys(Nor)-Ahx-His$_6$-NH$_2$ (5.16)



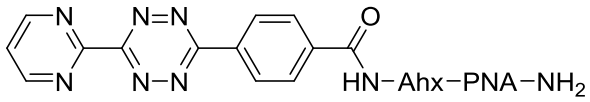
Aminomethyl polystyrene resin (2.5 g, 1.86 mmol, loading: 1.23 mmol/g) was pre-swollen in DCM, and Fmoc-Rink-OH linker coupled using Oxyma/DIC. Six Fmoc-His(Trt)-OH, Fmoc-Ahx-OH, Fmoc-Lys(Dde)-OH and 5-(11,12-didehydrodibenzo[*b,f*]azocin-5(6*H*)-yl)-5-oxopentanoic acid (**5.9**) were coupled as described earlier (**Section 7.2**). After Dde-deprotection (**Section 7.2.3**), *exo*-5-

norbornenecarboxylic acid was coupled (Oxyma/DIC) and the peptide was cleaved form the linker by addition of a solution of TFA:TIS:DCM (90:5:5, 2 × 90 min) and the product purified by HPLC (0.92 g, 0.6 mmol, 36%).

HPLC (method 1): t_R = 4.5 min (99% purity, ELSD)

MS (MALDI) *m/z*: calc. 1501.73, experimental 1503.6 [100%, $M+H^+$].

7.5.11. Synthesis of PNA-Tag (5.1)



Deprotection and coupling procedures are described in **Section 7.2**. Fmoc-Rink-LL-PS resin (20 mg, 5.89 μmol, 1 eq, or 30 mg, 5.44 μmol) was placed in a well on a 96-filter well plate with a bottom sealing matt, followed by stepwise coupling of PNA monomers (0.18 mmol, 3 eq) using a liquid handler and heating in an oven to 65 °C for 45 min. Aminohexanoic acid (0.18 mmol, 3 eq, DMF) and 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (0.18 mmol, 3 eq, DMSO/DCM 3:1) were coupled twice using Oxyma and DIC. PNAs were cleaved with TFA:water (95:5) and purified using HPLC (**Appendix 1** for sequences).

Table 7.1: Analysis of 100 PNA tags.

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
PNA001	4144.69	4150.82	8.3	3.0 (12)	**PNA051**	4119.68	4132.11	10.1	1.9 (8)
PNA002	4120.67	4125.89	8.1	2.2 (10)	**PNA052**	4117.65	4130.21	10.3	4.1 (17)
PNA003	4111.66	4118.65	9.0	0.8 (4)	**PNA053**	4069.63	4080.95	10.2	4.6 (19)
PNA004	4126.66	4132.78	9.1	1.9 (8)	**PNA054**	4093.64	4106.19	10.1	3.2 (13)
PNA005	4120.67	4126.72	9.0	1.2 (5)	**PNA055**	4111.66	4122.98	10.1	2.4 (11)

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
PNA006	4126.66	4129.37	9.2	1.0 (4)	**PNA056**	4117.65	4128.99	10.2	3.4 (15)
PNA007	4111.66	4113.12	9.0	1.8 (8)	**PNA057**	4108.64	4119.63	10.2	4.2 (17)
PNA008	4093.64	4096.04	9.1	1.1 (5)	**PNA058**	4126.66	4138.78	10.2	8.7 (39)
PNA009	4102.65	4104.62	9.2	0.8 (4)	**PNA059**	4090.62	4101.75	10.3	3.1 (13)
PNA010	4120.67	4123.92	9.0	3.2 (14)	**PNA060**	4102.65	4114.70	10.2	7.6 (34)
PNA011	4102.65	4106.06	9.1	3.2 (13)	**PNA061**	4117.65	4129.47	10.2	5.0 (21)
PNA012	4072.59	4077.37	10.5	1.7 (8)	**PNA062**	4102.65	4115.24	10.1	3.9 (16)
PNA013	4093.64	4099.66	10.1	1.6 (7)	**PNA063**	4117.65	4129.93	10.2	3.6 (15)
PNA014	4099.63	4104.12	10.3	3.7 (17)	**PNA064**	4126.66	4138.25	10.1	4.6 (19)
PNA015	4102.65	4108.78	10.0	2.1 (9)	**PNA065**	4108.64	4119.57	10.2	8.0 (36)
PNA016	4111.66	4117.71	10.0	3.5 (16)	**PNA066**	4102.65	4112.77	10.1	6.6 (30)
PNA017	4108.64	4114.69	10.3	1.9 (8)	**PNA067**	4144.69	4154.27	10.1	9.1 (40)
PNA018	4144.69	4149.31	10.1	4.4 (20)	**PNA068**	4108.64	4119.30	10.2	3.9 (17)
PNA019	4111.66	4116.81	10.0	4.5 (20)	**PNA069**	4102.65	4105.49 (-ve)	10.1	4.0 (17)
PNA020	4111.66	4117.29	9.8	3.1 (13)	**PNA070**	4102.65	4105.69 (-ve)	10.1	4.1 (18)
PNA021	4126.66	4132.14	10.2	6.9 (31)	**PNA071**	4102.65	4105.52 (-ve)	10.1	3.5 (14)
PNA022	4111.66	4114.84	10.0	5.2 (21)	**PNA072**	4126.66	4130.68 (-ve)	10.2	5.6 (25)
PNA023	4102.65	4106.06	10.1	5.9 (26)	**PNA073**	4135.67	4139.03 (-ve)	10.1	5.9 (24)
PNA024	4126.66	4131.78	10.2	1.6 (7)	**PNA074**	4135.67	4139.46 (-ve)	10.1	7.0 (31)
PNA025	4102.65	4107.53	10.1	2.0 (8)	**PNA075**	4108.64	4112.08 (-ve)	10.3	3.2 (13)
PNA026	4084.63	4095.28	10.2	1.6 (7)	**PNA076**	4111.66	4114.21	10.0	6.5 (29)
PNA027	4102.65	4113.57	10.1	3.9 (17)	**PNA077**	4120.67	4123.22 (-ve)	10.1	3.7 (17)

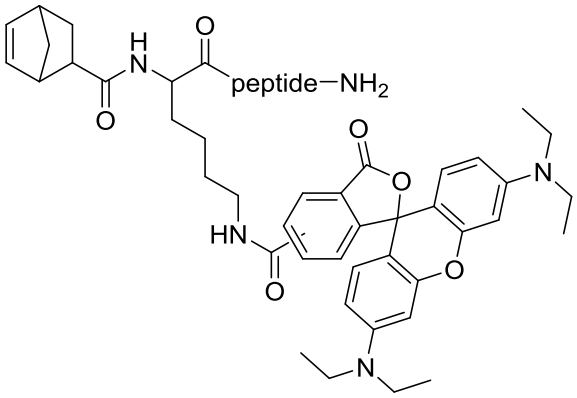
Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
PNA028	4144.69	4155.67	10.1	4.1 (17)	**PNA078**	4126.66	4130.74 (-ve)	10.1	4.0 (16)
PNA029	4084.63	4095.11	10.1	1.9 (9)	**PNA079**	4111.66	4112.69 (-ve)	10.0	3.7 (15)
PNA030	4075.62	4087.57	10.1	1.2 (5)	**PNA080**	4135.67	4138.71 (-ve)	10.1	6.3 (26)
PNA031	4126.66	4134.92	10.1	4.8 (21)	**PNA081**	4102.65	4106.62 (-ve)	10.1	2.7 (11)
PNA032	4111.66	4122.78	10.1	5.3 (22)	**PNA082**	4135.67	4139.93 (-ve)	10.1	3.5 (14)
PNA033	4084.63	4094.83	10.1	5.5 (25)	**PNA083**	4102.65	4103.87 (-ve)	10.0	6.3 (28)
PNA034	4111.66	4114.33	10.0	0.6 (3)	**PNA084**	4126.66	4130.53 (-ve)	10.1	8.3 (37)
PNA035	4107.64	- - -	- - -	0 (0)	**PNA085**	4144.69	4145.48	9.9	7.3 (30)
PNA036	4111.66	4122.66	10.1	0.9 (4)	**PNA086**	4120.67	4124.77 (-ve)	10.0	9.9 (44)
PNA037	4099.63	4109.25	10.4	1.7 (8)	**PNA087**	4111.66	4114.14 (-ve)	10.0	6.3 (28)
PNA038	4102.65	4112.58	10.1	5.6 (25)	**PNA088**	4108.64	4114.61	10.3	5.6 (25)
PNA039	4108.64	4120.34	10.3	2.1 (9)	**PNA089**	4120.67	4123.51	10.0	4.5 (19)
PNA040	4126.66	4138.59	10.2	2.1 (9)	**PNA090**	4111.66	4114.90 (-ve)	10.0	6.0 (27)
PNA041	4126.66	4137.76	10.1	6.7 (30)	**PNA091**	4111.66	4115.81	10.0	4.9 (22)
PNA042	4126.66	4137.73	10.2	3.5 (14)	**PNA092**	4135.67	4138.40	10.2	5.8 (26)
PNA043	4111.66	4123.53	10.0	6.5 (29)	**PNA093**	4144.69	4145.36	10.1	3.4 (14)
PNA044	4099.63	4110.14	10.2	3.8 (16)	**PNA094**	4117.65	4120.14	10.3	3.4 (14)
PNA045	4126.66	4137.07	10.2	6.8 (28)	**PNA095**	4111.66	4114.40	10.1	3.0 (12)
PNA046	4126.66	4137.72	10.2	3.0 (13)	**PNA096**	4135.67	4137.01	10.1	2.4 (10)
PNA047	4117.65	4129.04	10.1	1.7 (7)	**PNA097**	4102.65	4107.71	10.2	6.3 (28)
PNA048	4111.66	4123.14	10.1	3.7 (17)	**PNA098**	4135.67	4138.82	10.1	2.0 (9)
PNA049	4108.64	4119.63	10.3	5.6 (25)	**PNA099**	4135.67	4138.43	10.1	6.3 (28)

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
PNA050	4117.65	4128.93	10.2	5.8 (26)	**PNA100**	4120.67	4124.17	10.0	6.1 (27)

* = The MALDI-TOF MS spectra have an error of between 0.01–0.3%, and all the masses quoted for the PNA are stated as seen in the spectra without correction/internal calibration (see **Chapter 5**, **Figure 5.2** for explanation). Masses are $[M+H]^+$ unless otherwise indicated.

** = analysed by method 4 using ELSD detector signal.

7.5.12. Peptide synthesis for LigLib 1 (5.8)



All peptides (**Appendix 2**) were synthesis on a peptide synthesiser, starting with Fmoc-Rink-PS (50 mg, 37 μmol). After synthesis of the sequences, a small amount was cleaved and analysed by ELSD and MALDI-TOF MS, before manual coupling of Fmoc-Ahx-OH, Fmoc-Lys(Dde)-OH **5.3** and exo-5-norbornenecarboxylic acid using Oxyma/DIC as coupling agents at 60 °C for 30 min. After selective Dde-deprotection using hydroxylamine hydrochloride/imidazole, 5-(6) tetraethyl carboxyrhodamine was coupled using Oxyma/DIC for 18 h. Each member was cleaved under standard conditions (**Section 7.2.5**), followed by purification by HPLC and lyophilisation to yield each member as a red-pink solid.

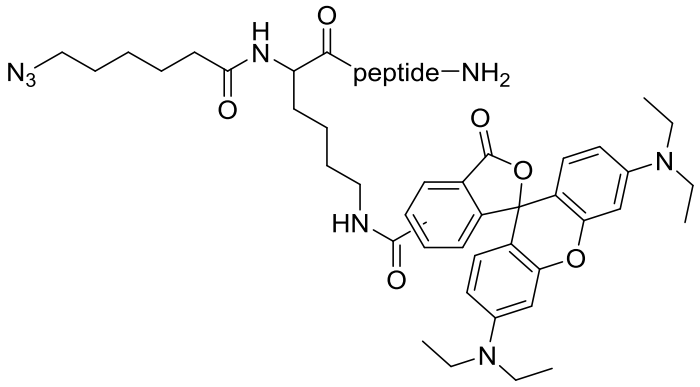
Table 7.2: Analysis and purify of synthesised peptides for **LigLib 1**.

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
Pep001	2591.17	2594.9	9.1/9.3	2.8 (3)
Pep002	2367.18	2370.7	9.2/9.4	1.9 (2)
Pep003	2675.33	2679.1	8.8/9.0	3.8 (4)
Pep004	2619.32	2623.0	9.6/9.8	5.4 (6)
Pep005	2608.34	2612.4	9.7/10.0	5.1 (5)
Pep006	2651.43	2655.2	8.5/8.7	1.3 (1)
Pep007	2385.24	2388.8	8.9/9.1	4.5 (5)
Pep008	2552.39	2556.1	8.5/8.7	3.6 (5)
Pep009	2679.42	2683.2	8.9/9.1	2.3 (2)
Pep010	2475.27	2479.0	10.6	1.4 (2)
Pep011	2499.24	2502.9	9.1/9.2	7.0 (8)
Pep012	2623.27	2627.0	9.0/9.2	5.1 (5)
Pep013	2666.47	2670.3	9.8/9.9	11.2 (11)
Pep014	2748.35	2752.2	9.1/9.3	1.8 (2)
Pep015	2653.23	2657.0	9.5/9.7	4.5 (5)
Pep016	2541.29	2544.9	8.8/9.0	2.8 (3)
Pep017	2833.66	2838.5	8.6/8.7	2.9 (3)
Pep018	2422.19	2425.8	9.3/9.5	16.3 (18)
Pep019	2241.08	2244.6	9.0/9.2	5.9 (7)
Pep020	2654.31	2658.1	9.4/9.6	2.5 (3)

* = The MALDI-TOF MS spectra have an error of between 0.1–0.2%, and all the masses quoted for the peptide are stated as seen in the spectra without correction/internal calibration (see **Chapter 5**, **Figure 5.2** for explanation). Masses are $[M+H]^+$ unless otherwise indicated.

** = analysed by method 3 using 530 nm detection; the two peaks correspond to the different isomers of 5-(6) tetraethyl carboxyrhodamine which were separated during purification.

7.5.13. Peptide synthesis for LigLib 2 (5.17)



To the synthesised peptides (**Section 7.5.12**), 6-azidohexanoic acid **3.3** was coupled using Oxyma/DIC at 60 °C for 30 min. After Dde-deprotection using hydroxylamine hydrochloride/imidazole, 5-(6) tetraethyl carboxyrhodamine was coupled using Oxyma/DIC for 18 h. Each member was cleaved using standard conditions (**Section 7.2.5**), followed by purification by HPLC and lyophilisation to yield each member as a red-pink solid.

Table 7.3: Analysis and purify of synthesised peptides for **LigLib 2**.

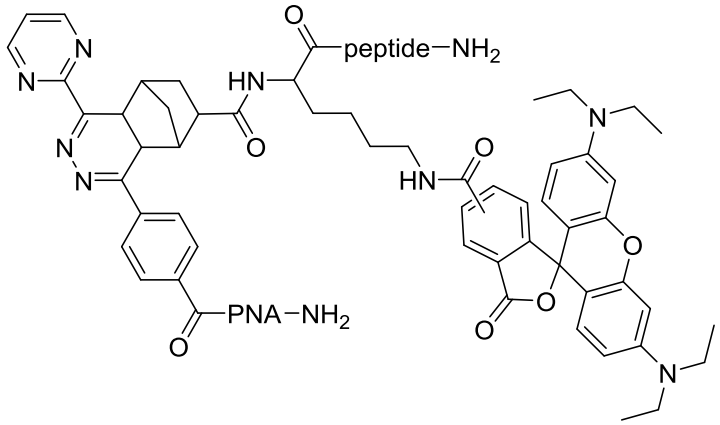
Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
Pep024	2678.43	2682.5	8.0/8.1	6.59 (7)	**Pep043**	2553.35	2557.3	8.2/8.4	7.57 (8)
Pep025	2605.25	2609.3	8.6	4.76 (5)	**Pep044**	2455.16	2459.7	7.8/8.0	6.54 (7)
Pep026	2648.34	2652.4	8.7	2.28 (2)	**Pep045**	2680.19	2684.5	8.5	4.78 (5)
Pep027	2618.27	2622.4	9.1	2.19 (2)	**Pep046**	2603.39	2607.7	8.7	3.00 (3)
Pep028	2733.37	2737.6	8.3/8.4	3.53 (3)	**Pep047**	2597.34	2601.3	7.9/8.1	4.97 (5)
Pep029	2635.22	2639.8	7.8/8.0	5.20 (5)	**Pep048**	2674.25	2678.2	9.1/9.2	3.19 (3)
Pep030	2664.47	2668.6	8.4/8.6	7.52 (8)	**Pep049**	2460.27	2464.1	8.8/9.1	17.56 (19)
Pep031	2567.24	2571.3	8.5/8.6	6.87 (7)	**Pep050**	2349.18	2353.0	8.6	7.20 (8)
Pep032	2457.24	2461.2	7.7/8.0	4.39 (5)	**Pep051**	2542.22	2546.5	8.7/9.1	7.93 (8)
Pep033	2642.47	2646.7	7.3/7.9	5.67 (6)	**Pep052**	2624.34	2628.4	8.5/	8.35 (9)
Pep034	2397.22	2401.2	8.0/8.2	5.35 (6)	**Pep053**	2534.24	2538.2	8.4	3.47 (4)
Pep035	2671.43	2675.5	8.4/8.6	4.78 (5)	**Pep054**	2692.16	2696.2	8.5	5.96 (6)
Pep036	2707.40	2711.8	7.8/8.0	6.78 (7)	**Pep055**	2718.37	- - -	- - -	0 (0)
Pep037	2583.33	2587.5	8.7/8.8	6.94 (7)	**Pep056**	2639.34	2643.7	9.4	1.05 (1)
Pep038	2670.37	2674.6	7.7/7.9	10.10 (10)	**Pep057**	2629.34	2633.5	7.8/8.0	8.10 (8)
Pep039	2628.44	- - -	- - -	0 (0)	**Pep058**	2736.34	2741.4	8.3	4.30 (4)
Pep040	2675.23	- - -	- - -	0 (0)	**Pep059**	2638.39	2642.4	8.4/8.6	9.59 (10)

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*	*Sq. No.*	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)*
Pep041	2712.33	2716.7	8.0/8.2	6.96 (7)	**Pep060**	2567.32	2571.5	8.9	6.20 (7)
Pep042	2675.40	2679.8	8.0	2.56 (3)					

* = The MALDI-TOF MS spectra have an error of between 0.1–0.2%, and all the masses quoted for the peptides are stated as seen in the spectra without correction/internal calibration (see **Chapter 5**, **Figure 5.2** for explanation). Masses are $[M+H]^+$ unless otherwise indicated.

** = analysed by method 3, 530 nm detection; the two peaks correspond to the different isomers of 5-(6) tetraethyl carboxyrhodamine which were separated during purification.

7.5.14. Synthesis of LigLib 1



For the ligation, the TZ-PNA (1 eq, **Section 7.5.11**) was dissolved in water/acetonitrile (0.8 mL) containing 0.1% TFA, and the peptide (1.5 eq, **Section 7.5.12**) was added. The reaction was heated to 50 °C for 18 h. The conjugate was purified by HPLC, and the fractions concentrated under reduced pressure and lyophilised to yield the conjugates as purple/red solids.

Table 7.4: Analysis of 20 members of **LigLib 1**.

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)****	*PNA and peptide used*
Lig001	6707.85	6715.2	10.5	1.25 (26)	PNA001 Pep001
Lig002	6459.85	6465.7	10.8	0.84 (36)	PNA100 Pep002

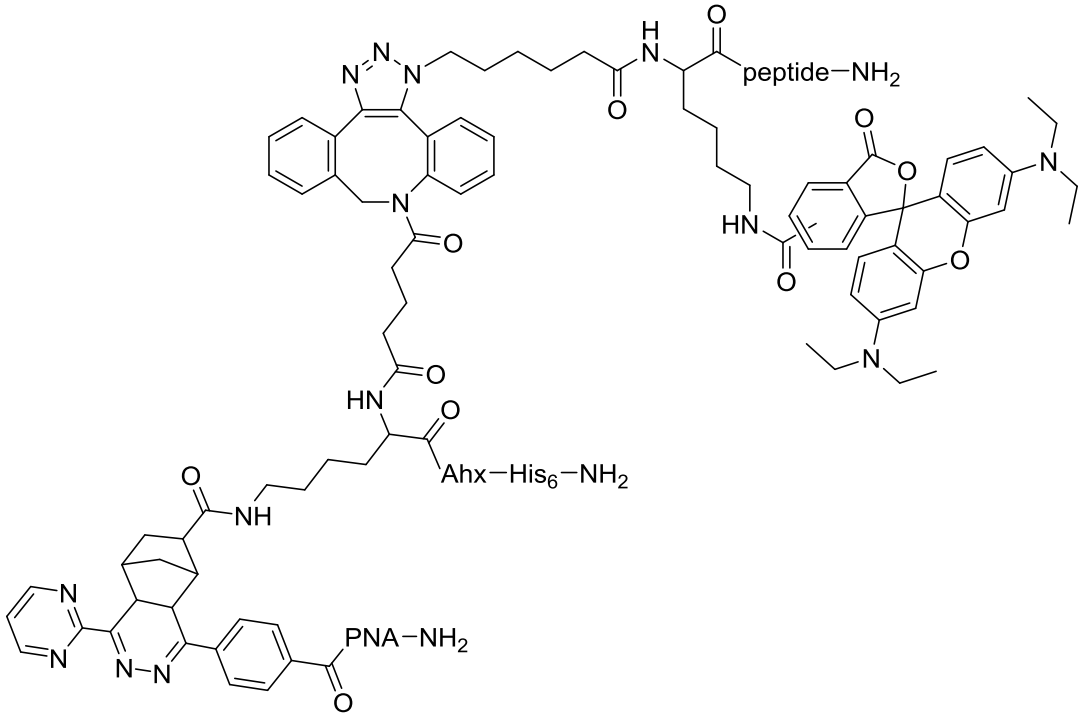
Lig003	6758.99	6768.1	10.5	1.00 (76)	PNA003 Pep003
Lig004	6702.98	6710.2	11.3	1.67 (34)	PNA095 Pep004
Lig005	6707.00	6715.5	11.9	1.67 (35)	PNA078 Pep005
Lig006	6735.05	6743.9	10.5	3.62 (75)	PNA087 Pep006
Lig007	6468.90	6475.8	10.7	2.05 (48)	PNA079 Pep007
Lig008	6627.04	6634.0	10.6	2.41 (62)	PNA071 Pep008
Lig009	6754.07	6762.6	10.9	0.96 (28)	PNA083 Pep009
Lig010	6564.92	- - -	- - -	0 (0)	PNA094 Pep010
Lig011	6582.89	6589.8	11.0	2.81 (50)	PNA091 Pep011
Lig012	6697.92	6705.1	10.9	2.89 (66)	PNA081 Pep012
Lig013	6759.13	6790.6	11.9	2.23 (37)	PNA077 Pep013
Lig014	6856.02	6864.3	11.0	1.41 (43)	PNA098 Pep014
Lig015	6745.90	6753.0	11.5	3.36 (64)	PNA089 Pep015
Lig016	6648.96	6656.2	10.7	0.81 (21)	PNA096 Pep016
Lig017	6941.32	6948.7	10.5	1.72 (45)	PNA082 Pep017
Lig018	6502.82	6509.8	11.4	2.64 (44)	PNA088 Pep018
Lig019	6321.71	6329.7	11.1	0.80 (16)	PNA075 Pep019
Lig020	6770.99	6779.2	11.4	0.79 (19)	PNA093 Pep020

* = The MALDI-TOF MS spectra have an error of between 0.08–0.2%, and all the masses quoted for the conjungates are stated as seen in the spectra without correction/internal calibration (see **Chapter 5**, **Figure 5.2** for explanation). Masses are $[M+H]^+$ unless otherwise indicated.

** = analysed by method 4, 530 nm detection.

*** = yield based on PNA used.

7.5.15. Synthesis of LigLib 2



For the ligation, the peptide (1.5 eq, **Section 7.5.13**) was dissolved in water/ACN (1:1, 0.8 mL) containing 0.1% TFA, and the His-tag (2 eq, **Section 7.5.10**, **5.16**) was added. The reaction was heated to 50 °C for 18 h, after which azide-PS scavenger resin pre-swollen in DCM was added (10 mg) and heated at 50 °C for 8 h, followed by removal of the resin by filtration and the addition of TZ-PNA (1 eq, **Section 7.5.11**). The reaction was heated to 50 °C for 18 h. After this the conjugate was directly purified by HPLC, and the fractions concentrated under reduced pressure and lyophilised to yield the conjugates as purple/red solids.

Table 7.5: Analysis of 34 members of **LigLib 2**.

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)****	*PNA and peptide used*
Lig021	8287.82	8311.2 $[M+Na]^+$	6.6	0.54 (5)	PNA092 Pep024
Lig022	8214.65	- - -	- - -	0 (0)	PNA080 Pep025
Lig023	8230.70	- - -	- - -	0 (0)	PNA068 Pep026
Lig024	8200.63	- - -	- - -	0 (0)	PNA057 Pep027

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)****	*PNA and peptide used*
Lig025	8318.75	- - -	- - -	0 (0)	PNA032 Pep028
Lig026	8244.61	4145.7 $[(M+2Na)/2]^+$	6.8	1.88 (16)	PNA073 Pep029
Lig027	8273.87	8299.0 $[M+Na]^+$	7.8	2.27 (16)	PNA074 Pep030
Lig028	8143.61	4072.2 $[(M+2H)/2]^+$	7.5	1.11 (8)	PNA066 Pep031
Lig029	8057.63	8080.8 $[M+Na]^+$	6.5	1.08 (10)	PNA072 Pep032
Lig030	8227.86	8252.7 $[M+Na]^+$	6.5	2.16 (18)	PNA090 Pep033
Lig031	7973.59	- - -	- - -	0 (0)	PNA023 Pep034
Lig032	8253.79	- - -	- - -	0 (0)	PNA049 Pep035
Lig033	8292.79	- - -	- - -	0 (0)	PNA043 Pep036
Lig034	8183.71	- - -	- - -	0 (0)	PNA041 Pep037
Lig035	8288.78	- - -	- - -	0 (0)	PNA067 Pep038
Lig036	8312.71	8339.0 $[M+Na]^+$	7.1	2.03 (15)	PNA045 Pep041
Lig037	8293.81	- - -	- - -	0 (0)	PNA018 Pep042
Lig038	8171.76	8171.8 $[M+H]^+$	6.7	2.31 (16)	PNA085 Pep043
Lig039	8040.54	8063.5 $[M+Na]^+$	6.6	2.48 (20)	PNA076 Pep044
Lig040	8256.56	8282.3 $[M+Na]^+$	7.2	1.38 (12)	PNA038 Pep045
Lig041	8188.77	8213.0 $[M+Na]^+$	7.0	0.7 (8)	PNA019 Pep046
Lig042	8188.71	8211.8 $[M+Na]^+$	6.6	1.7 (15)	PNA050 Pep047
Lig043	8274.64	- - -	- - -	0 (0)	PNA031 Pep048
Lig044	8054.67	8077.3 $[M+Na]^+$	7.2	0.57 (3)	PNA086 Pep049

Sq. No.	*Calc. MS g/mol*	*Found MS m/z**	*ELSD-HPLC min***	*Yield mg (%)****	*PNA and peptide used*
Lig045	7949.56	7972.1 $[M+Na]^+$	6.9	2.36 (18)	PNA021 Pep050
Lig046	8118.59	- - -	- - -	0 (0)	PNA060 Pep051
Lig047	8224.73	8249.7 $[M+Na]^+$	7.1	2.19 (13)	PNA084 Pep052
Lig048	8119.62	- - -	- - -	0 (0)	PNA022 Pep053
Lig049	8301.56	- - -	- - -	0 (0)	PNA099 Pep054
Lig050	8257.75	- - -	- - -	0 (0)	PNA028 Pep056
Lig051	8211.70	4106.7 $[(M+2H)/2]^+$	7.0	2.08 (13)	PNA065 Pep057
Lig052	8294.69	8319.6 $[M+Na]^+$	6.7	1.24 (12)	PNA033 Pep058
Lig053	8238.78	8264.2 $[M+Na]^+$	7.1	1.55 (9)	PNA058 Pep059
Lig054	8143.69	8167.5 $[M+Na]^+$	7.1	0.52 (4)	PNA097 Pep060

* = The MALDI-TOF MS spectra have an error of between 0.001–0.04%, and all the masses quoted for the conjugates are stated as seen in the spectra without correction/internal calibration (see **Chapter 5**, **Figure 5.2** for explanation). Masses are $[M+H]^+$ unless otherwise indicated.

** = analysed by method 4 using 530 nm detection.

*** = yield based on PNA used.

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Appendix

Appendix

I. PNA Sequences

Melting temperature (T_m) describe the temperature at which half of a probe (in this case the PNA) sequence will be hybridised onto the target sequence (in this case DNA). To calculate these theoretical temperatures, the 4 + 2 method (Wallace-Ikatura rule)[145,146] was used. In this method, for adenine and thymine residue counts 2 °C, and every guanine and cytosine counts 4 °C (**Equation 1**).

$$T_m = 2\,℃(A+T) + 4\,℃(G+C) \qquad (1)$$

Table A.1: Table of PNA sequences synthesised.

PNA Nº	***C*-Terminal**										***N*-Terminal**				T_m /°C
T_m Calc. for 1	4	2	2	2	2	4	2	2	2	2	4	2	2	2	34
1	C	T	A	A	A	C	A	T	A	A	C	T	A	A	34
2	A	C	T	A	A	C	A	T	C	A	T	A	C	A	36
3	C	T	A	A	C	A	T	T	C	T	A	A	A	C	36
4	C	T	A	A	A	C	T	T	C	T	A	A	T	A	34
5	C	T	A	A	C	T	A	A	T	A	A	C	A	C	36
6	C	T	A	A	A	C	T	A	A	C	A	T	T	T	34
7	T	A	A	C	C	A	T	T	T	A	A	C	C	A	36
8	C	A	T	T	C	A	T	T	A	C	T	T	A	C	36
9	C	A	T	T	C	T	A	C	A	A	C	T	A	T	36
10	A	C	A	T	C	T	A	A	A	C	A	T	A	C	36
11	C	T	A	A	T	T	T	C	A	C	T	A	A	C	36
12	T	T	T	C	T	T	T	C	T	T	T	C	T	T	34
13	A	A	T	C	A	C	T	T	C	T	T	C	A	T	36
14	T	T	T	C	A	T	A	T	C	A	T	T	C	T	34
15	A	C	T	T	A	A	C	T	A	C	T	T	A	C	36
16	A	C	T	A	C	T	A	A	A	C	T	T	A	C	36
17	A	C	T	A	T	T	T	C	A	C	T	A	T	T	34
18	A	C	T	A	A	A	C	T	C	A	T	A	A	A	34

PNA Nº	*C*-Terminal													*N*-Terminal	T_m /°C
19	C	A	T	A	A	A	C	T	C	A	T	T	C	A	36
20	C	T	A	A	C	A	T	A	A	C	T	T	C	A	36
21	A	C	T	A	A	A	C	T	C	T	A	A	T	T	34
22	A	C	T	A	A	C	T	T	A	A	C	T	C	A	36
23	T	T	T	C	A	A	C	T	C	T	A	A	A	C	36
24	C	A	T	A	A	C	A	T	A	A	C	T	T	T	34
25	A	C	T	T	C	T	A	A	A	C	T	T	C	A	36
26	A	C	T	T	T	T	T	C	A	C	A	T	C	T	36
27	T	T	T	C	A	A	C	T	C	A	T	A	A	C	36
28	A	A	C	T	A	T	T	A	C	A	A	A	C	A	34
29	T	T	T	C	T	T	T	C	C	T	A	A	A	C	36
30	C	A	T	T	T	T	T	C	T	T	T	C	C	A	36
31	C	T	A	A	T	T	T	C	A	A	C	T	A	A	34
32	C	A	T	C	T	A	A	T	C	A	A	A	C	T	36
33	T	T	T	C	C	T	A	A	T	T	T	C	A	C	36
34	T	A	C	T	T	A	A	C	C	A	T	A	C	A	36
35	A	C	T	T	T	A	C	T	T	A	C	T	T	A	34
36	T	A	A	C	T	A	A	C	A	C	T	T	A	C	36
37	T	A	A	C	T	T	T	C	T	A	C	T	T	T	34
38	T	A	A	C	A	A	C	T	T	T	T	C	A	C	36
39	T	A	C	T	A	C	A	T	T	A	C	T	T	T	34
40	A	T	T	A	C	A	A	C	A	C	T	T	A	T	34
41	T	A	A	C	T	A	C	T	C	A	T	A	T	A	34
42	C	A	A	A	T	A	T	C	A	C	T	T	T	A	34
43	A	C	A	T	T	A	C	T	C	A	T	A	A	C	36
44	C	A	T	A	T	T	T	C	T	T	T	C	T	A	34
45	A	A	C	T	C	A	T	T	A	A	C	T	T	A	34
46	A	C	A	T	A	A	C	T	T	A	C	T	T	A	34
47	T	A	A	T	A	C	T	T	C	A	T	T	C	A	34
48	T	A	A	T	A	A	C	T	C	T	A	C	A	C	36
49	C	A	T	T	C	A	T	T	T	A	A	C	T	T	34
50	C	A	T	T	T	A	C	T	A	C	T	A	T	A	34
51	T	A	A	C	A	A	C	T	A	C	T	A	A	C	36
52	T	A	A	C	A	C	T	T	C	A	T	A	T	T	34
53	A	C	A	T	C	T	T	C	T	A	C	T	C	T	38
54	T	A	C	T	T	A	A	C	T	T	T	C	A	C	36

PNA Nº	*C*-Terminal													*N*-Terminal	T_m /°C
55	C	A	A	T	C	A	A	T	A	A	C	T	C	T	36
56	T	T	T	C	A	C	T	A	C	A	T	A	T	A	34
57	C	A	T	T	A	C	T	A	T	T	T	C	T	A	34
58	C	A	T	C	A	T	T	A	C	A	A	T	T	A	34
59	T	T	T	C	A	C	T	T	T	T	T	C	T	A	34
60	T	A	C	T	C	T	A	A	T	A	A	C	C	T	36
61	A	C	T	T	C	T	A	A	T	A	C	T	T	A	34
62	T	A	A	C	C	T	A	A	T	A	C	T	C	T	36
63	A	C	A	T	C	A	T	A	T	T	T	C	A	T	34
64	C	A	T	T	C	A	T	C	A	T	T	A	A	A	34
65	T	T	T	C	C	A	T	T	C	T	A	A	T	A	34
66	C	A	T	C	T	A	T	C	A	T	A	T	C	A	36
67	C	T	A	A	A	C	A	T	T	A	A	C	A	A	34
68	T	T	T	C	T	T	T	C	T	A	A	C	A	A	34
69	C	T	A	C	A	C	T	T	A	C	A	T	T	A	36
70	A	C	T	A	T	A	C	T	T	A	A	C	C	T	36
71	T	A	C	T	T	A	A	C	T	A	A	C	C	T	36
72	C	T	A	A	T	A	A	C	C	T	A	A	T	T	34
73	T	A	A	C	A	A	C	T	T	A	A	C	T	A	34
74	A	C	T	T	T	A	A	T	C	A	A	A	C	A	34
75	A	C	A	T	T	T	T	C	T	A	A	C	T	T	34
76	C	A	T	A	A	A	T	C	T	A	C	T	C	A	36
77	C	A	T	A	C	A	T	A	A	C	A	T	A	C	36
78	A	C	T	A	T	A	T	C	T	A	T	C	A	A	34
79	T	A	C	T	A	C	T	A	C	A	T	A	A	C	36
80	C	A	A	T	C	A	A	T	C	T	A	A	T	A	34
81	T	A	A	C	A	A	T	C	T	T	T	C	A	C	36
82	T	A	C	T	C	A	T	A	C	A	A	T	A	A	34
83	A	C	A	T	T	A	T	C	A	C	T	T	C	A	36
84	T	A	A	C	T	A	T	C	T	A	C	T	A	A	34
85	A	C	T	A	C	A	A	A	C	T	A	A	T	A	34
86	T	A	A	C	A	A	T	C	A	C	T	A	C	A	36
87	A	C	T	A	C	A	T	T	C	A	T	A	A	C	36
88	C	T	A	A	C	A	T	T	T	A	T	C	T	T	34
89	T	A	T	A	C	A	A	C	A	A	C	T	C	A	36
90	A	A	T	C	T	A	C	T	T	A	A	C	A	C	36

PNA Nº	*C*-Terminal													*N*-Terminal	T_m /°C
91	C	A	T	T	C	A	A	T	A	C	T	A	A	C	36
92	C	A	T	A	A	C	T	A	C	A	A	A	T	T	34
93	C	A	A	A	C	A	T	A	A	A	T	C	T	A	34
94	T	A	A	T	A	A	C	T	C	A	T	C	T	T	34
95	A	A	T	C	A	C	A	T	C	A	T	T	C	A	36
96	C	A	A	A	T	T	T	C	A	A	T	C	A	A	34
97	T	A	T	C	A	C	A	T	A	C	A	T	C	T	36
98	A	A	C	T	T	A	C	T	A	A	T	C	A	A	34
99	C	A	A	T	A	C	T	T	T	A	A	C	A	A	34
100	C	A	A	T	C	T	A	A	A	C	A	T	C	A	36

II. Peptide Sequences

Table A.2: Kinases of interest with corresponding phosphorylation sides and substrate's.

Sequence Nº	Kinase	Target Sequence	Substrate	PO_3 Side
1	Abl	LGQEEDVYHTVDDDE	BCAP	Y513-p
2	Abl	SVTAPSPYAQPSSTF	p63	Y149-p
3	Abl	GEGYKKKYQQVDEEF	PDGFRb	Y970-p
4	Ack	AYQSRDYYNFPLALA	AR	Y363-p
5	ALK	QVPQQPTYVQALFDF	Grb2	Y160-p
6	ALK	SKRKGHEYTNIKYSL	SHP-2	Y546-p
7	Arg	DHGAEIVYKSPVVSG	tau iso8	Y394-p
8	AurA	KGKFGNVYLAREKQS	AurA	Y148-p
9	Brk	RLIKEDVYLSHDHNI	Brk	Y342-p
10	Brk	AVVNLINYQDDAELA	CTNNB1	Y142-p
11	Btk	QSHVEDLYVEGLPEG	TFII-I iso2	Y357-p
12	BVR	ESSSHEDYIRQFLNA	BVR	Y83-p
13	CDK7	HEVVTLWYRAPEILL	CDK2	Y168-p
14	CdkL5	NNANYTEYVATRWYR	CdkL5	Y171-p
15	CK2-A1	DWGLAEFYHPGQEYN	CK2-A1	Y182-p
16	CSFR	DPEGGVDYKNIHLEK	CSFR	Y699-p
17	CSFR	KYKQKPKYQVRWKII	CSFR	Y546-p
18	CSK	FTATEPQYQPGENL-	Yes	Y537-p
19	CTK	YTATEGQYQQQP---	Lyn	Y508-p
20	EGFR	ISLDNPDYQQDFFPK	EGFR	Y1172-p
21	EGFR	QLTFALRYLNFFTKA	PCNA	Y211-p
22	EGFR	TENDDDVYRSLEELA	VAV2	Y142-p
23	EphA2	TYVDPHTYEDPNQAV	EphA2	Y594-p
24	EphA2	QLKPLKTYVDPHTYE	EphA2	Y588-p
25	EphA4	TYVDPFTYEDPNQAV	EphA4	Y602-p
26	EphA4	LNQGVRTYVDPFTYE	EphA4	Y596-p
27	EphB3	VYIDPFTYEDPNEAV	EphB3	Y614-p
28	ERK7	EDQAVTEYVATRWYR	ERK7	Y177-p
29	Etk	TKVELETYKQTRQGL	RUFY1	Y389-p
30	FAK	AAFRLFKYGVQLYKN	Grb7	Y338-p
31	FAK	KDNFDSFYSEVAELG	Trio	Y2796-p
32	Fer	RQEDGGVYSSSGLKQ	Fer	Y714-p

Sequence Nº	Kinase	Target Sequence	Substrate	PO_3 Side
33	Fer	EAKILKQYDHPNIVK	Fer	Y615-p
34	Fes	REEADGVYAASGGLR	Fes	Y713-p
35	Fes	RPSFSTIYQELQSIR	Fes	Y811-p
36	FGFR1	DIHHIDYYKKTTNGR	FGFR1	Y654-p
37	FGFR2	TLTTNEEYLDLSQPL	FGFR2	Y769-p
38	FGFR3	DVHNLDYYKKTTNGR	FGFR3	Y648-p
39	FGFR4	LLAVSEEYLDLRLTF	FGFR4	Y754-p
40	FLT3	TGSSDNEYFYVDFRE	FLT3	Y589-p
41	FLT3	KEHNFSFYPTFQSHP	FLT3	Y726-p
42	FRK	NKDKANRYFSPNFKV	PTEN	Y336-p
43	Fyn	PPVPNPDYEPIRKGQ	CD3E	Y188-p
44	Fyn	EHGSQGTYSNTKENG	MAP2 iso3	Y67-p
45	Fyn	EEEKEETYDDIDGFD	SKAP55	Y232-p
46	Hck	VLNSHDLYQKVAQEI	ELMO1	Y216-p
47	Hck	RVIEDNEYTAREGAK	Hck	Y411-p
48	HER2	SPAFDNLYYWDQDPP	HER2	Y1221-p
49	HER2	PTAENPEYLGLDVPV	HER2	Y1248-p
50	HER2	GAVENPEYLTPQGGA	HER2	Y1196-p
51	IGF1R	YASVNPEYFSAADVY	IGF1R	Y980-p
52	IGF1R	DIYETDYYRKGGKGL	IGF1R	Y1166-p
53	InsR	DPKEDPIYDEPEGLA	Dok1	Y362-p
54	InsR	NEDADENYFINEEDE	PIK3R3	Y341-p
55	ITK	IRSEENIYTIEENVY	TIM3	Y265-p
56	Jak2	WDQEAQIYELVAQTV	ARHGEF1	Y738-p
57	Jak2	TSDEKVDYVQVDKEK	Gab2	Y643-p
58	Jak3	IETDKEYYTVKDDRD	JAK1	Y1035-p
59	Jak3	LPLDKDYYVVREPGQ	Jak3	Y981-p
60	Kit	INGNNYVYIDPTQLP	Kit	Y570-p
61	Kit	DHAEAALYKNLLHSK	Kit	Y703-p
62	Lck	LIIEDPYYGNDSDFE	ACP1	Y133-p
63	Lck	NLQERRKYLKHRLIV	STAT2	Y690-p
64	Lck	GEAPSNIYVEVEDEG	TSAd	Y305-p
65	LRRK2	GFPAIRDYHFVNATE	LRRK2	Y1485-p
66	Lyn	TILTEVNYEVSNKDD	Casp8	Y448-p
67	Lyn	NEPKKKKYAKEAWPG	NIPP-1	Y335-p

Sequence Nº	Kinase	Target Sequence	Substrate	PO_3 Side
68	Mer	KIYSGDYYRQGRIAK	Mer	Y754-p
69	PDGFRa	RLSADSGYIIPLPDI	PDGFRa	Y1018-p
70	PDGFRa	RVDSDNAYIGVTYKN	PDGFRa	Y988-p
71	PDGFRb	ERKEVSKYSDIQRSL	PDGFRa	Y754-p
72	PDGFRb	LWQKKPRYEIRWKVI	PDGFRb	Y562-p
73	PKR	QLAAKLAYLQILSEE	PKR	Y162-p
74	Ret	ARTTSQLYDAVPIQS	PDK1	Y9-p
75	Ret	TPSDSLIYDDGLSEE	Ret	Y1029-p
76	Ret	AQAFPVSYSSSGARR	Ret	Y687-p
77	Ret iso2	TWIENKLYGRISHAF	Ret iso2	Y586-p
78	Src	VPVDPATYGQFYGGD	gelsolin	Y465-p
79	Src	ELLEQQKYTVTVDYW	IKK-beta	Y199-p
80	Src	LPGKPKVYVQDILRQ	iNOS	Y1055-p
81	Src	WQNYRQAYSPRRGRS	Trap150	Y118-p
82	Syk	EENADDSYEPPPVEQ	BLNK	Y96-p
83	Syk	EEDGEPDYVNGEVAA	LAB	Y233-p
84	Syk	LRADENYYKAQTHGK	Syk	Y526-p
85	TGFBR2	KGRFAEVYKAKLKQN	TGFBR2	Y259-p
86	TGFBR2	AKGNLQEYLTRHVIS	TGFBR2	Y336-p
87	TIE2	TYVNTTLYEKFTYAG	TIE2	Y1108-p
88	TrkA	LAQAPPVYLDVLG--	TrkA iso2	Y785-p
89	TrkB	SFDAILYYYQSGGRI	Kv1.3	Y162-p
90	TXK	RYVLDDEYVSSFGAK	TXK	Y420-p
91	TXK	KIQVKALYDFLPREP	TXK	Y91-p
92	Tyk2	NHIGHTGYLNTVTVS	RACK1	Y194-p
93	Tyk2	VPEGHEYYRVREDGD	Tyk2	Y1055-p
94	VEGFR-1	DYNSVVLYSTPPI--	VEGFR-1	Y1333-p
95	VEGFR-1	VQQDGKDYIPINAIL	VEGFR-1	Y1169-p
96	VEGFR-2	RFRQGKDYVGAIPVD	VEGFR-2	Y951-p
97	VEGFR-2	EEAPEDLYKDFLTLE	VEGFR-2	Y996-p
98	Wee1	EKIGEGTYGVVYKAR	CDK2	Y15-p
99	ZAP70	EEEGAPDYENLQELN	LAT iso2	Y226-p
100	ZAP70	SLDGSREYVNVSQEL	LAT iso2	Y191-p